

EXPERIMENTAL STUDIES ON THE ACUTE THROMBOGENICITY OF BIOLOGIC AND SYNTHETIC VASCULAR PROSTHESES

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To

Margaretha, Jens, Mattias and Hanna
who coped with both my absence and
my presence.

This thesis is based on the following papers:

- I. Uptake and inactivation of thrombin by the fresh, glutardialdehyde or heparin treated human umbilical cord vein endothelium.

C-G Björck, R Larsson, P Olsson and U Rothman.
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- II. In vitro evaluation of a biologic graft surface. Effect of treatment with conventional and low molecular weight heparin.

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- V. In vivo evaluation of the acute thrombogenicity of the modified human umbilical vein and autologous artery.

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VII. Thromboxane production in umbilical vein grafts.

P Saldeen, CO Esquivel, C-G Björck, D Bergqvist and T Saldeen.

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VIII. The effect of inhibition of thromboxane synthesis in experimental thrombosis and haemostasis.

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INTRODUCTION

The progress of vascular surgery during the 20th century has been intimately coupled to the increasing use of different vascular prostheses (Dale, 1978; Wesolowski, 1978). Much effort has been paid to defining and developing the "ideal vascular graft", the aim being to resemble as closely as possible the normal artery with its multiple and complicated properties (Szilagyi, 1982). In regions of the vascular tree with large diameters (>6 mm) and high flow-rates, efforts have been successful, and various modifications of Dacron grafts function satisfactorily when replacing aorta and iliac arteries (Sauvage et al. 1978). However, when femoral, popliteal or even tibial/peroneal arteries need to be revascularized, bypass procedures give variable results and are less successful (Creech et al., 1957; Donaldsson & Mannick, 1980; Kacoyannis et al., 1981; LiCalzi & Stansel, 1982).

In the late 1940s, Kunlin (1949) first described the use of a vein bypass to the popliteal artery, and since then the autologous saphenous vein has generally been considered the best available graft when dealing with small caliber arteries (<6 mm) and low flow-rates (Szilagyi, 1978a). During the last decade two graft materials have distinguished themselves as good alternatives in cases of good run-off, namely expanded polytetrafluorethylene (PTFE) and modified human umbilical vein (Dardik Biograft) (Campbell et al., 1979; Buchardt Hansen et al., 1980; Aamold et al., 1981; Bergan et al., 1982a; Dardik et al., 1982; Lee et al., 1982; Eickhoff et al., 1983). However, when arteriosclerotic lesions are wide-spread resulting in a defective distal arterial bed, the autologous vein is still the graft of choice (Edwards & Mulherin, 1980; Hobson et al., 1980; Weisel et al., 1981; Bergan et al., 1982b; Cranley & Hafner, 1982; Julian et al., 1982; ; Yeager et al., 1982;). Because of varicosities, thrombophlebitis or too small diameter, the vein may be unavailable or unsuitable. Moreover, harvesting the autologous vein is a time-consuming procedure (Abbott 1977), and may



eventually lead to postoperative complications, such as haematoma formation, extensive swelling and infection. Improved diagnostic techniques such as digital subtraction angiography (Goldstone, 1984) and pre-bypass intraoperative angiography (Flanigan et al., 1982; King et al., 1984) have also resulted in an increasing demand for femorodistal bypass procedures, and thus there exists a great need for an easily available and reliable alternative to the autologous vein graft. This is true also for the rapidly increasing field of coronary-bypass surgery.

The thrombogenicity of a particular graft material, i.e. its tendency to activate platelets and coagulation enzymes and thus initiate thrombus formation, is one of the major determinants of the early performance of the graft. Efforts to improve outcome have been made both experimentally, using animal models to study the thrombogenicity of artificial surfaces in vivo (Sauvage et al., 1979; Carson et al., 1981) and clinically, in the form of trials comparing different graft modifications and adjunctive regimes. In analogy to Virchow's triad (1856), methods to improve graft patency can be classified into regimes aiming at 1) modification of the graft surface, i.e., reducing its thrombogenicity, 2) increasing the blood flow through the graft or 3) decreasing the reactivity of the constituents of the blood to the graft surface. Modification of the surface has been attempted by the use of biologic tissue, e.g. mandril-grown grafts (Sparks, 1972; Perloff et al., 1981), arterial regeneration over absorbable prostheses (Bowald et al., 1979; Greisler, 1982) and endothelial seeding (Stanley et al., 1982). Another way of reducing the surface thrombogenicity is the use of different methods of supplying the surfaces with a heparin layer. A number of other factors such as porosity, compliance, flexibility and durability also influence graft performance and must be considered when methods to improve outcome are being discussed (Wesolowski, 1978). Methods of increasing blood flow include haemodilution or dextran administration (Shah et al., 1980) and creation of arteriovenous

fistulae (Blaisdell et al., 1966; Campbell & Harris, 1984). With the objective of reducing the reactivity of blood constituents, substances which affect the coagulation system as a whole, e.g. heparin, dicumarol or warfarin, and platelet activity, e.g. ASA, non-steroidal anti-inflammatory agents or selective thromboxane inhibitors have been used (Murray, 1940; Heimbecker, 1977; Carson et al., 1981; Green et al., 1982; McEnany et al., 1982; ; Goldman et al., 1983a; Hanson & Harker, 1983).

The main purpose of the present investigation was to evaluate in vitro and in vivo the surface thrombogenicity of different vascular graft materials, (Biograft, PTFE and polyurethane (PU)) and to compare them with the "ideal" vascular grafts, the autologous vein and artery. The effect of surface modification using different methods of applying heparin treatment was also studied. Finally, the effect of systemic administration of a selective thromboxane synthesis inhibitor on the acute thrombogenicity of Biografts in vivo was evaluated.

MAJOR CONTRIBUTIONS TO THE EVOLUTION OF VASCULAR SURGERY

Although a number of vascular procedures were successfully performed before the introduction of arteriography, this diagnostic technique soon evolved into a fundamental building block in the evolution of vascular surgery. The contributions of dos Santos et al. (1929), who introduced the technique of translumbar aortography, and of Seldinger of Sweden (1953), who developed the technique of catheterization, improved arteriography to become "the cornerstone of vascular surgery" (Foster, 1974).

The problem of intra- and postoperative thrombosis was, at least partly, solved by the discovery of heparin (Howell & Holt, 1918) and its introduction in clinical practice in general (Crafoord, 1936; Jorpes, 1936) and in vascular surgery in particular (Murray, 1940). The frequency of postoperative thrombo-embolic complications after vascular as well as general surgery, was further decreased by the development and clinical use of different dextrans, with effects on the hemostatic system, particularly platelets, as well as on blood flow (Gelin et al., 1961; Bergentz, 1978).

In 1888, Rudolph Matas treated a case of brachial artery aneurysm with aneurysmorrhaphy, which he reported in 1902 and in 1897 JB Murphy successfully performed the first clinical end-to-end arterial suture, using an invagination technique. During the first decade of the 20th century, Carrel and Guthrie laid the foundations of modern arterial surgery, working experimentally with vascular grafts, particularly homologous vein grafts (Carrel, 1906). In 1912 Carrel was awarded the Nobel prize for Physiology and Medicine "in recognition of his works on vascular suture in the transplantation of blood vessels and organs" (Schuck et al., 1951). In 1906 Goyanes performed the first successful interposition of a venous graft for an aneurysmal popliteal artery segment in man. A year later, Erich Lexer (1907) used a saphenous vein segment to bridge a defect after resection of

a traumatic false aneurysm in the axilla. Another important contribution was the work of Bertram Bernheim who in 1913 presented a monograph on vascular surgical techniques. Later he reported the successful resection of a syphilitic popliteal artery aneurysm and its replacement by a 12 cm long segment of the saphenous vein (Bernheim, 1916).

DEVELOPMENT OF VASCULAR GRAFTS

Synthetic grafts

At the beginning of the fifties a large variety of materials had been tried as arterial substitutes. These were all in the form of hollow, solid tubes made of paraffin-lined silver, glass, vitallium or polyethylene, and the flow surfaces were designed so as to resemble the smooth inner surface of the normal artery. All of these, however, failed due to thrombosis or haemorrhage from the suture lines. In 1952 Voorhees and associates found that a silk suture projecting into the lumen of a dog's heart became covered with a thin layer of tissue. They postulated that the interior of a cloth tube might be covered similarly, while its interstices became blocked with fibrin and platelets. Adopting this new principle of development of the flow surface of the graft after blood contact they successfully used tubes of Vinyon N in dogs and in 1953 they inserted the first synthetic graft in man. This discovery led to intense research using different materials, and a further improvement of these materials was made when Edwards and Tapp (1955) found that shrinking the tubes allowed them to be bent without kinking. In 1957 a committee appointed by the Society for Vascular Surgery in the USA presented a survey of current vascular graft materials (Creech et al., 1957). They pointed out that large diameter arteries with high flows enabled the use of a variety of materials, but noted that no currently available graft material met suitable criteria for peripheral small-vessel replacement, although the better ones were dacron and orlon.

During the last decade much research has been focused on synthetic materials for small artery replacement and the best material available, appears to be the expanded polytetrafluoroethylene (PTFE), which was introduced about ten years ago (Matsumoto et al., 1973; Campbell et al., 1975). It is a chemically inert material, with an electronegative and hydrophobic surface (Campbell et al., 1979). The micropores of the graft allow ingrowth of surrounding tissue but are not permeable to blood and thus it needs no pre-clotting. Results for femoropopliteal and femorotibial bypasses using PTFE-grafts are in general good, but in cases of low flow and high peripheral resistance, this synthetic graft is still inferior to the autologous vein (Hallett et al., 1981; Bergan et al., 1982b).

Biologic grafts

The only true biologic grafts in clinical practice are the autologous vein and artery, the latter being used only in rare instances (Ehrenfeld et al., 1982). Other grafts belonging to this group are merely of biologic origin, and most of their native characteristics are destroyed by treatments applied after harvesting.

The autologous saphenous vein. Although saphenous vein segments were used already during the first decade of this century, it was only after Kunlin described the method of femoro-popliteal bypass (1949) that the use of the autologous vein was revived. Linton (1955) popularized it in the United States, and its usefulness in trauma situations was confirmed during the Korean War (Hughes, 1958). During the last decades, important contributions have been made by Szilagyí in his studies of the biologic fate of and atherogenesis in venous bypass grafts (Szilagyí et al., 1973; Szilagyí, 1978b). His findings indicate that the autologous vein still has certain limitations as an arterial substitute, although it seems to be the best alternative.

Arterial homografts. The first attempts to preserve arteries for later use as arterial substitutes were made by Carrel, but subsequent efforts were not successful until 1949, when Gross and associates reported 15 successful homologous arterial grafts used for coarctation of the aorta and tetralogy of Fallot. A variety of techniques for preserving human arteries were developed after the report of Gross (Hufnagel, 1952). These were studied by Hallén in Sweden (1955), who found freezing and freeze-drying to be superior to preservation in a nutrient medium, because the former methods allowed longer periods of storage. Freeze-drying was more complicated but had the advantage of producing grafts that could be kept at room temperature and were easier to transport. However, the incidence of degenerative changes resulting in thrombosis, and aneurysmal formation was found to be too high to be acceptable, and led to the abandonment of the material only about 15 years after its introduction (Szilagyi et al., 1957; DeWeese et al., 1959).

Bovine heterografts. Bovine arterial grafts were introduced in 1956 (Rosenberg et al.). Bovine carotid arteries were subjected to ficin digestion of surrounding tissues resulting in pure collagenous tubes, which were then tanned with dialdehyde starch. These grafts gained widespread popularity in femoropopliteal bypass surgery and were used as the second choice after autologous veins for almost 20 years. However, as time passed, it became apparent that degenerative changes resulting in aneurysmal dilatations, infections and thrombosis were far too common (Dale, 1976) and during the late 70s, their use was confined almost entirely to arterio-venous fistulae for angio-access. A recent study comparing bovine heterografts to PTFE grafts as AV-fistulae showed no statistically significant difference but a review of impressions of the graft materials indicated a decided preference for PTFE grafts (Hunt et al., 1983).

A new modification of a bovine carotid heterograft has recently been developed, the manufacturers claiming that the

preparation method gives elastic properties and wettability much like those of normal arteries (Erasmi et al., 1983). The performance of this prosthesis (Solcograft P) in animal experiments is reported to be good, but follow-up periods have been short and no human implantations have so far been reported.

Mandrill-grown grafts. In 1961 Eiken and Nordén reported on a technique of growing small artery fibrocollagenous grafts by implanting plastic rods adjacent to the carotid arteries in dogs. Better known, however, are the later die-grown grafts designed by Sparks (1968). In the first publication he described the development of tissue dies by means of which the patient could grow his own arterial grafts. In 1970 he reported good results of long-term animal and clinical experiments (Sparks 1970). However, follow-up studies revealed very poor long-term performance with a patency of 26% in the femoropopliteal and 0% in the femorotibial positions (Hallin & Sweetman, 1976). The complications included poor maturation of the grafts, early thrombosis, postoperative haemorrhage and aneurysm formation. A modern modification of the mandrill-grown grafts was presented a few years ago (Perloff et al., 1981). Those grafts were grown subcutaneously in sheep, supported by a polyester mesh and tanned in glutaraldehyde. Preliminary results were promising, but clinical follow-up periods were short and another group, working with the same type of prosthesis, has reported discouraging results (Parsonnet, 1981).

In addition to the "biologic" grafts mentioned above several, often anecdotal, reports of the use of biologic tissue have appeared, such as autologous fascia (Pratt, 1958), pericardium (Sako, 1951) and skin, either full-thickness or split-skin (Horton et al., 1956; Sandblom & Dahlbäck, 1957).

Human umbilical vein grafts. The human umbilical vein was first used for long bypass grafts in 1951 (Anzula et al.) when six 20-24 cm long segments of native human umbilical

veins were inserted into dogs. All, however, thrombosed within 2 hours. In 1960 Nabseth et al. implanted fetal umbilical arteries into dog aorta. The grafts were preserved either in sterile 20% homologous serum at 4°C or by freezing at -70°C. Eighteen of 31 grafts functioned for more than one month, but upon removal 11 of these showed aneurysmal dilatation and further inspection revealed degenerative changes. The same disappointing results were encountered by Yong and Eiseman (1962), who anastomosed human umbilical veins, both fresh and alcoholpreserved, to canine aortae. Immediate postoperative mortality was high and only one graft of nine remained patent until the sacrifice 3 months later. The problem of the host antigen-antibody reaction was thus not overcome by preservation in alcohol.

In 1973 Dardik and associates reported the use of human umbilical veins as aortic substitutes in the baboon. They found that aneurysms developed three weeks after implantation, but continued to study different modifications of the veins and found that glutaraldehyde fixation at room temperature provided an adequate introduction of cross-links to resist degradation in biological fluids (Baier et al., 1976). Further experimental evaluation showed glutaraldehyde to be superior to dialdehyde starch, and tanned umbilical vein segments were successfully implanted in baboons (Dardik & Dardik, 1976). Early clinical experience demonstrated the need for preventing graft thrombosis by obviating the presence of excess or residual aldehyde polymers (Dardik et al., 1977). Preoperative rinsing with heparinized saline was thus introduced. The graft was also provided with an outer polyester mesh, giving a tensile strength approximately eightfold that of normal arterial systolic pressure (Baier et al., 1976). Further clinical studies by both Dardik and other authors have demonstrated excellent early results, comparable to those of autologous saphenous vein, but definitive long-term results are not yet available (Buchardt Hansen et al., 1980; Boontje, 1982; Dardik et al., 1982; Hirsch et al., 1982; Lee et al., 1982; Myhre et al., 1982).

"Biologic approaches" to vascular grafting

Arterial regeneration. Insertion of a polyglactin 910 mesh as a patch or a tubular graft into the thoracic aortae of pigs has been shown to result in a regeneration of tissue resembling that of normal arteries in nearly all respects (Bowald et al., 1979; Bowald et al., 1980). The arterial tissue was completely endothelialized after 20 days and the polyglactin mesh had disappeared after 40 days, the new wall appearing to retain sufficient strength throughout the observation period. In 1982, Greisler reported arterial regeneration over a mesh of polyglycolic acid inserted as 24 mm long tubular grafts into the rabbit aorta.

Endothelial seeding. Enzymatically derived endothelial cells added to the blood used to preclot small-diameter Dacron grafts have been demonstrated to form a monolayer endothelial covering in animal experiments and thus improve patency as compared to unseeded control grafts (Stanley et al., 1982). PTFE grafts used as thoracoabdominal bypasses in dogs developed an endothelial covering after being flushed with blood mixed with autologous, cultured endothelial cells (Graham et al., 1982). The results of endothelial seeding have recently been thoroughly reviewed (Stanley, 1984).

BLOOD INTERACTIONS WITH THE VESSEL WALL

The intact endothelial lining is non-thrombogenic, but following arteriotomy and anastomosing to a vascular graft, subendothelial structures such as basement membrane and collagen to which platelets readily adhere and initiate thrombus formation are exposed. Endothelial damage following manipulation may also be present in autologous vein grafts and, in addition, arteriosclerotic lesions impair endothelial function. It is thus of interest to consider the anti-thrombotic characteristics of the endothelium when discussing the thrombogenicity of artificial materials. Of special interest is arachidonic acid metabolism and the production of prostacyclin (PGI_2) and thromboxane A_2 (TxA_2). Blood compatibility of the endothelium is also influenced by factors such as the superficial presence of glucosaminoglycans (Kirk, 1959; Isuku & Murata, 1973; Hatton et al., 1978) and antithrombin III (Chan & Chan, 1979), specific binding sites for thrombin (Awbrey et al., 1979; Lollar & Owen, 1980) and heparin (Glimelius et al., 1978), thrombin inhibiting capacity (Dryjski et al., 1982) and production of plasminogen activators (Todd, 1959; Isacsson & Nilsson, 1972; Ljungnér & Bergqvist, 1983).

Arachidonic acid metabolism

Arachidonic acid is the precursor of the prostaglandins as outlined in fig. 1 (Moncada et al., 1978; Moncada & Vane, 1979). Arachidonic acid is oxygenated and cyclized by cyclooxygenase to form the cyclic endoperoxides PGG_2 and PGH_2 . The end products thus formed are qualitatively and quantitatively different, possibly depending upon the major function(s) of the tissue involved. Thus, in endothelial cells, prostacyclin synthetase metabolizes most of the endoperoxide to prostacyclin (PGI_2). It has been demonstrated that the endothelial cells can also obtain endoperoxides from stimulated platelets (Marcus et al., 1981). In platelets thromboxane synthetase metabolizes the endoperoxide to thromboxane A_2 (TxA_2) (Hamberg et al., 1975). PGI_2 is

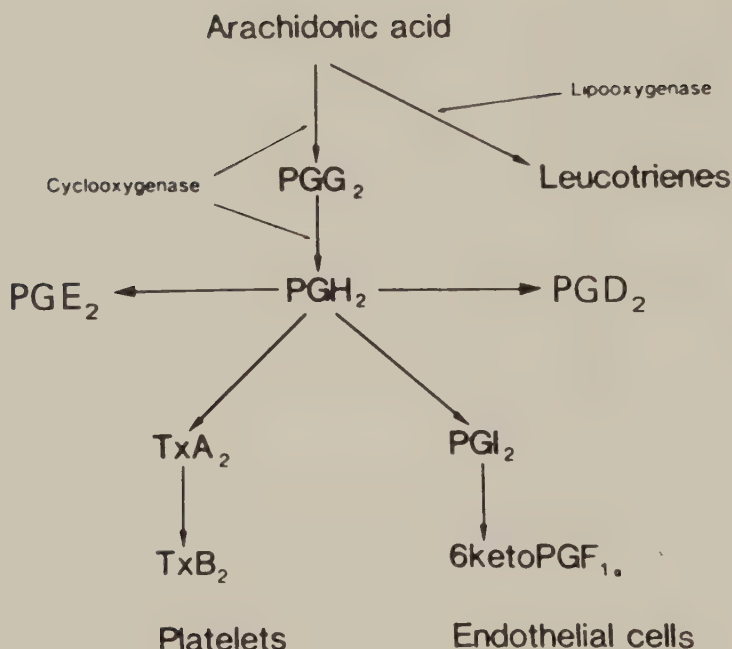


Fig. 1. The arachidonic acid metabolism.

the most potent antiaggregatory agent known, whereas TxA_2 promotes platelet aggregation (Gryglewski et al., 1978). PGI_2 also acts as a vasodilator, whereas TxA_2 has a vasoconstricting ability. Both PGI_2 and TxA_2 are very unstable in the circulation and are metabolized to the stable end products $6\text{-keto-PGF}_{1\alpha}$ and TxB_2 respectively, which can be assayed radioimmunologically (Karmali et al., 1980; Belch et al., 1983). The endoperoxide PGH_2 can also be metabolized to PGD_2 , PGE_2 and PGF_2 , the first of which acts as an inhibitor of platelet aggregation, while at least PGE_2 probably acts pro-aggregatory.

The lipoxygenase pathway results in the formation of leukotrienes, some of which are believed to serve as cyclo-oxygenase activators (Lands, 1979).

During the last few years of intense research on prostaglandins, prostacyclin and thromboxane production has been detected in different tissues such as smooth muscle tissue (Baenziger et al., 1979; Ody et al., 1982), arterialized venous grafts (Eldor et al., 1981), dacron arterial prostheses (Clagett et al., 1982) and vascular and lung tissue (Saldeen & Saldeen, 1983). Cultured endothelial cells produce both TxA_2 and PGI_2 (Ingerman-Wojenski et al., 1981) and human vessel walls increase production of both TxA_2 and PGI_2 following mechanical trauma (Metha et al., 1982). Recently a significantly higher production of PGI_2 in umbilical veins and arteries as compared to placental and uterine vessels was observed (Kawano & Mori, 1983).

In summary, there are probably multiple factors governing the interaction of circulatory platelets with the vascular endothelium. The formation of different prostaglandin endoperoxides and the balance between prostacyclin production by the endothelium and thromboxane production by platelets provides one approach to preventing thrombosis by using substances interfering with the metabolism of arachidonic acid.

All artificial surfaces currently used as vascular grafts are thrombogenic. Not even the autologous saphenous vein is completely non-thrombogenic, since the harvesting and storage technique may damage the endothelial lining, which is the ultimate prerequisite for blood compatibility (Ramos et al., 1975; Yoder et al., 1982). To be able to evaluate different methods of reducing the thrombogenicity of vascular grafts, it is essential to discuss the events following contact between blood and artificial surfaces.

Protein adsorption

When a foreign surface comes in contact with blood proteins are immediately adsorbed (Baier & Dutton, 1969). A large number of proteins of different molecular weight, size, conformation and concentration compete for adsorption sites on the surface. The resulting protein layer is important in determining the thrombogenic characteristics of the surface. Thus, surfaces which preferentially adsorb albumin will be less thrombogenic than those adsorbing gammaglobulin and fibrinogen, which will actively cause clotting (Vroman & Adams, 1967; Lee et al., 1974). It is not yet known in detail which proteins are adsorbed to different surfaces (Brash, 1981). Red blood cells seem to play a role in restricting protein adsorption (Brash & Uniyal, 1976). A characteristic of importance for the adsorption of proteins is the "wettability" of the surface. "Nonwetable" surfaces are called hydrophobic, meaning that they do not allow water and aqueous fluids to spread out but instead form separated drops having a non-zero contact angle (Brash, 1977). Hydrophobic surfaces adsorb substantially more proteins, particularly albumin, than hydrophilic ("wetable") ones (Uniyal & Brash, 1982).

Surface interactions with blood cells

Besides protein adsorption after blood-surface contact platelets and other blood elements adhere (Evans & Mustard,

1968; Salzmann, 1971; Salzmann et al., 1977). Plasma contains several proteins, which seem to function as adhesive proteins for platelets. According to experimental evidence, these are also present in secretory pools within platelets. Such proteins are fibrinogen, the von Willebrand factor, fibronectin and thrombospondin (Mosher, 1981). Albumin, on the other hand, inhibits the accumulation of platelets on the surface (Lee et al., 1974). Adherent platelets undergo release reaction leading to further aggregation of platelets (Baumgartner et al., 1976).

Adhesion of leukocytes is also associated with the prior adsorption of a monolayer of proteins such as fibrinogen, IgG and Factor XII (Vroman et al., 1977). Leukocytes, other white and also red cells interact with the surface and may significantly contribute to thrombogenesis (Keller, 1981). Procoagulant activity has been demonstrated in both leukocytes and lymphocytes (Forbes, 1981), and leukocytes also release thromboxane A_2 , which promotes platelet aggregation (Goldstein et al., 1978). Red blood cells may enhance platelet aggregation mechanically or by release of their ADP content (Goldsmith, 1971).

Surface interaction with coagulation factors

The intrinsic pathway of blood coagulation is activated when human blood is exposed to an artificial surface. Four plasma proteins are involved in normal contact activation reactions: Factor XII, Factor XI, plasma prekallikrein and high molecular weight kininogen (Griffin, 1981). Thrombin and antithrombin also interact with artificial surfaces and it has been proposed that multiple types of binding sites which are specific for certain plasma proteins may exist on various artificial surfaces (Chuang et al., 1979).

The release of different substances from platelets adhering to the surface is followed by an acceleration of the formation of a meshwork of fibrin trapping red and white blood cells. Thus, thrombus formation may spread over the entire

surface, especially during conditions of low flow and turbulence (Sauvage et al., 1973).

EVALUATION OF THROMBOGENICITY

A number of tests have been devised for evaluating the thrombogenicity of various graft surfaces. Most of these depend on measuring the activation of the coagulation system components such as different platelet tests and blood coagulation tests (Forbes, 1981). A number of experimental models to evaluate the thrombogenic characteristics of graft surfaces, both in vitro and in vivo, have been developed. Attempts to image grafts placed in humans with radioactively-labelled platelets have also been reported.

In vitro models

Most current models for testing of graft materials in vitro are designed as artificial circulations, determining different platelet variables and often relating these to findings post-perfusion with scanning electron microscopy (SEM). Rajah and Hamlin described an artificial circulation, designed to simulate the blood pressure and flow-rate in normal healthy femoral arteries (Rajah et al., 1977; Hamlin et al., 1978). Using this method they examined PTFE, woven Dacron and glutaraldehyde-treated human femoral artery (HFA) for their effects on platelet function, adhesion and aggregation. Dacron grafts were found to produce the largest reduction in platelet count and also induced platelet adhesion and aggregation responses more than HFA and PTFE; the latter of which exhibited the least effect on platelet functions. SEM studies also showed the least platelet adherence to PTFE and the greatest to Dacron grafts. Thus, the findings indicated that PTFE had a lower thrombogenicity than Dacron. Using the same model, Goldman et al. (1983b) compared the modified human umbilical vein (Biograft) and PTFE. Graft segments were perfused for 45 minutes with heparinized blood containing human ^{113}In -labelled platelets, and platelet deposition on the graft surfaces as well as platelet count and change in threshold aggregation responses were recorded. Platelet deposition was significantly greater on the Biograft, which was confirmed by SEM studies of the surfaces.

Dolgov et al. (1983) used a similar in vitro model studying PTFE, Biograft, the Bakulev bioimplant (another biologic prosthesis produced from umbilical veins) and, in addition, the Solcograft P. They used the native and denuded umbilical vein as a reference, defining thrombogenic and adhesive indices for the different graft materials, by the use of a quantitative SEM-analysis. Indices for PTFE, Biograft and the Bakulev bioimplant approximated those of the non-damaged umbilical vein, while the Solcograft exhibited considerably higher thrombogenic characteristics.

In vitro tests also include stress/strain measurements, assessment of mechanical properties and graft porosity and measurements of infrared spectra and electron spectroscopy (Lyman et al., 1977). These methods are of importance particularly in developing different synthetic polymers. Their use precedes more specific thrombogenicity determinations, and hence they will not be discussed further.

In vivo animal models

Thrombogenicity studies using animal implantation models have frequently been presented in the literature. The variables examined include evaluation of mechanical properties and postimplantation histology (Lyman et al., 1977). Also assessment of patency and of neointimal fibrous hyperplasia development in long-term implantations (Oblath et al., 1978a) have been reported as well as attempts to imagine platelet deposition on graft surfaces by the use of radioactively labelled platelets (Dewanjee et al., 1978; Roedersheimer et al., 1980). Hanson et al. (1980) used a baboon arterio-venous shunt model, ^{51}Cr -labelled platelets and ^{125}I -labelled fibrinogen, studying platelet consumption per unit area of graft surfaces. This was found to depend strongly on the type of material, but did not vary with shunt flow-rate, circulating platelet count or total shunt surface. The surfaces studied included acrylic polymers, polyurethanes and expanded PTFE. The platelet consumption induced by artificial surfaces was also demonstrated to

result in the production of microemboli, this process depending strongly on the physicochemical properties of the prosthetic material. Christenson et al. (1981a) studied PTFE grafts in dogs, using ^{111}In -labelled platelets and found platelet deposition to be dependent on the blood flow, when this was lower than a threshold value of about 50 ml/min. In a later study using ^{111}In -labelled platelets and PTFE grafts in dogs, it was demonstrated that the possibility to interpret the nuclear images of synthetic grafts depended on the duration between establishing flow in the graft and the administration of labelled platelets (Megerman et al., 1983).

Sauvage et al. (1979) used an acute canine model, implanting different types of grafts in the carotid arteries and perfusing them at controlled low flow-rates. They evaluated the thrombus free surface (TFS) and defined the thrombotic threshold velocity (TTV) for a particular graft surface as the flow-rate at which the TFS score falls below 50%. Thrombogenicity as derived from these controlled flow studies turned out to be more predictive of the long-term implantation success of a graft than were one-week implantation results. Grafts studied were ranked in order of increasing thrombogenicity: non-crimped Dacron, PTFE, crimped Dacron, Biograft. Roedersheimer et al. (1980) compared platelet accumulation in Biografts and autogenous vein grafts using chromium-labelled platelets and found Biografts to exhibit a high tendency to accumulate platelets and, in addition, expressed the opinion that the handling characteristics of the Biograft were suboptimal.

Carson et al. (1981) employed the flow-reduction model in sheep using 4 mm PTFE grafts, demonstrating a low percentage of thrombus free surface at flows of 50 ml/min or lower. At 24 hours, all grafts occluded at low flows. Comparing PTFE and noncrimped polypropylene-supported filamentous velour knitted Dacron (PPSFV) grafts with the inner diameters of 4 mm, Kenny et al. (1980) found the latter graft to perform

better than PTFE. The PPSFV grafts were preclotted according to a specific regime utilizing heparin.

In vivo evaluation of grafts in humans

Studies showing accelerated consumption of platelets in patients with vascular prostheses provide indirect evidence that platelets may be important in this process (Harker et al., 1977). McCollum studied platelet and fibrinogen kinetics using ^{51}Cr -labelled platelets and ^{125}I -labelled fibrinogen in patients who had received Dacron aorto-femoral grafts, finding that platelet kinetics were equal to their preoperative values first at 9 months postoperatively, while fibrinogen consumption reached normal values 6 months postoperatively (McCollum et al., 1981). The authors concluded Dacron grafts to be thrombogenic for about 9 months postoperatively.

More direct evidence of increased platelet deposition on prosthetic grafts compared with normal vessels can be derived from imaging techniques using ^{111}In -labelled platelets. This method has been used to study platelet deposition at vascular access sites (Ritchie et al., 1982) and in implanted arterial grafts (Goldman et al., 1982; Pumphrey et al., 1983; Goldman et al., 1983c). Using ^{111}In -labelled platelets, comparison between autologous vein, PTFE and velour Dacron grafts used as femoropopliteal bypasses showed autologous vein to be markedly less thrombogenic. Assessment of platelet survival failed to differentiate among the three graft materials (Goldman et al., 1982). A thrombogenicity index calculated as the mean daily rise in the ratio of radioactivity in the graft to the contralateral thigh was shown to be of predictive value concerning subsequent graft thrombosis (Goldman et al., 1983c).

CLINICAL EVALUATION OF SMALL DIAMETER ARTERIAL SUBSTITUTES

The ultimate goal of all efforts to evaluate and decrease the thrombogenicity of vascular graft surfaces is the development of satisfactory prostheses to bypass obstructed arteries of small diameter (less than 6 mm). Thus, a critical survey of clinical results with present graft materials may yield information influencing the design and properties of future vascular prostheses.

The autologous artery has for several years been considered the ideal graft material (Wylie, 1965). Due to unavailability its use is, however, restricted primarily to replacement of the renal artery by the internal iliac artery and other rare instances (Stoney & Wylie, 1980; Ehrenfeld et al., 1982).

The autologous saphenous vein gives the best results in femoropopliteal and femorodistal bypass surgery. Patencies exceeding 60% at five years are common (Cutler et al., 1976; DeWeese & Rob, 1977; Maini & Mannick, 1978; Naji et al., 1978; Reichle et al., 1979; Donaldson & Mannick, 1980; Kacoyanis et al., 1981). Thus, the advantages of the autologous vein are well documented, but certain limitations in its use have been observed. Szilagyi et al. (1979) summarized these and stated that poor veins, i.e., those with diameters less than 4 mm, with damages (mechanical or phlebitic) or inappropriate branching, may be inferior to synthetic grafts and should not be used. This opinion is contradicted by the results of Sonnenfeld & Cronstrand (1980), who used all veins with a diameter of more than 2 mm and found useful veins in 97% of patients undergoing femoropopliteal bypass surgery. The percentage availability of the saphenous vein in this study is very large compared to that reported by most other authors, finding the vein to be absent or useless in 10-40 % of the cases. However, in the Swedish study graft patency was found to be independent of the size and quality

of the vein, 5-year patency exceeding 80%.

The technique of in situ saphenous vein bypass is advocated by some authors (Leather et al., 1982; Corson et al., 1984), one advantage with this being the maintenance of the vasa vasorum, which may play an important role in preserving the original functions of the endothelium.

Dacron prostheses and bovine heterografts are at present not frequently being used in femoropopliteal/-distal bypass and are generally considered to possess a higher thrombogenicity than both the saphenous vein and the newer synthetic and biologic grafts. Infection is also a serious problem when developing in connection with these grafts and aneurysm formation is a common complication when bovine grafts are used. In a recent retrospective clinical study comparing Dacron and PTFE in femoropopliteal bypass procedures for severe lower limb ischaemia results with Dacron were significantly better (van Det et al., 1984). However, this study showed extremely low patency-rates for PTFE, which may explain the unwanted finding.

PTFE and umbilical vein grafts have gained increasing popularity during the last few years and clinical results are beginning to equal those achieved with the saphenous vein (Burnham et al., 1978; Campbell et al., 1979; Dardik et al., 1978; Haimov et al., 1979; Buckhardt Hansen et al., 1980; Gupta & Veith, 1980; Dardik et al., 1982). However, the results of different studies are difficult to compare due to different criteria of patient selection and there is often inadequate characterization of patient material, particularly as regards the quality of run-off vessels.

Comparative studies

Several retrospective studies comparing saphenous vein with other graft types have been published during the last years. In some of these studies, the observation periods are

too short to allow any differences to be demonstrated (Veith et al., 1978; Hobson et al., 1980). Other studies show similar patency-rates for vein and the artificial materials in cases of good run-off, but most authors agree that the autologous vein is superior in cases of poor run-off (Julian et al., 1980; Aamold et al., 1981; Wiesel et al., 1981). Comparing PTFE, Biograft and autologous vein, Cranley & Hafner (1982) found Biograft to be an acceptable alternative to saphenous vein and PTFE to give poor results when passing the knee joint. The PTFE findings were confirmed by Yeager et al. (1982). In a recent review, Bergan et al. (1982a) concluded that both PTFE and Biograft are inferior to vein in distal bypasses when revascularization is for limb salvage and in low flow, high peripheral resistance situations. The importance of the graft material was further stressed by Ricco et al. (1983), studying different variables such as patient age, graft material, type of bypass, bypass outflow, presence or absence of pedal arterial arches and degree of preoperative ischemia. The only obvious factor in graft failure was the graft material, saphenous vein performing better than PTFE.

The results of two randomized prospective studies have been published during the last years. Bergan et al. (1982b) compared PTFE and autologous vein and found no differences in the above-knee position, whereas autologous vein performed significantly better at 2 1/2 years when the distal anastomosis was made infrapopliteally. Eickhoff et al. (1983) compared PTFE and Biograft in a study in which all distal anastomoses were made below the knee. The one-year results demonstrated significantly better patency rates for Biograft.

In summary, two graft materials, PTFE and modified human umbilical vein (Biograft) at present seem to be the best alternatives to the autologous saphenous vein for femoropopliteal and femorodistal reconstructions, although some authors claim that the failure-rates with these alternatives

are so high that other autologous veins, e.g., the cephalic and basilic veins should be used as the first choice (Edwards & Mulherin, 1980). Comparisons between PTFE and Biograft are so far very few and no decided preference for one over the other can yet be stated, although the one published prospective randomized study favours the Biograft. Further clinical studies as well as experimental evaluation and attempts to decrease thrombogenicity are thus indicated. An interesting issue is the use of platelet antiaggregating substances, particularly those with a specific effect on thromboxane synthesis.

METHODS TO REDUCE THROMBOGENICITY

Methods to develop blood compatible vascular prostheses have aimed at producing graft materials with mechanical properties and surface characteristics resembling those of normal vessels. It is well documented that such mechanical properties as compliance, flexibility, porosity and durability are of great importance for the performance of vascular prostheses (Gozna et al., 1974; Baird & Abbott, 1977; Clark et al., 1978, Wesolowski, 1978; Walden et al., 1980) but, since this presentation deals mainly with the acute thrombogenicity, these matters will not be further discussed.

There are two main approaches to reduce the surface thrombogenicity: one aims at making the surface as nonreactive as possible to blood elements and the other tries to furnish the surface with an endothelial lining. The acute thrombogenicity is of critical importance in both groups, and it is interesting to consider surface properties of artificial materials and how these can be modified.

Hydrophobic/hydrophilic surfaces

Hydrophobic ("non-wettable") surfaces do not allow water to spread out or to be absorbed. They adsorb more albumin than hydrophilic surfaces, a fact of importance since albumin coating is believed to diminish platelet adhesion and aggregation. Hydrophilic ("wettable") surfaces, on the other hand, allow water to spread and also to become absorbed into the bulk of the material (Brash, 1977). Most polymers currently used as vascular conduits are of hydrophobic nature, e.g. PTFE and polyurethane.

Surface charge

Normal vascular intima has a negative net charge of -3 to -13 mV (Sawyer et al., 1953). Since platelets and other blood elements also have a negative charge, the vessel wall will repel them and this is probably a factor contributing to the antithrombotic nature of the intima. It is also known

that endothelial injury reverses the potential, making it strongly positive (Stoffey & Lee, 1969). Attempts have been made to reduce the charges of polymers by incorporating a fixed ratio of negative charges (Sharp et al., 1968). However, these surfaces rapidly adsorb fibrinogen and Factor XII, which may be activated and induce coagulation (Nossel et al., 1969). Sawyer et al. (1978a, 1978b) studied the effects of electric charges and introduced a glutaraldehyde treated collagen prosthesis with induced negative surface charge, which is currently the object of clinical trials. The modified human umbilical vein has a negative surface charge (-12 to -16 mV), as has PTFE.

Critical surface tension (CST)

The CST reflects the surface free energy of a particular material and can be correlated with the relative resistance or tendency to formation of thrombosis (Baier et al., 1976). Surfaces whose CST fall within the biocompatible range of 25 ± 5 dynes/cm tend to be thromboresistant. The CST of the human umbilical vein graft, like that of siliconized glass, falls within this range while polytetrafluoroethylene has a value of 18 dynes/cm (Brash, 1977). The pyrolytic carbon coated graft presented by Sharp et al. shows a CST of 28 dynes/cm after blood contact, and this, too, is within the biocompatible range (Sharp & Teague, 1978).

Heparin bonding

The natural vessel wall contains different glucosaminoglycans and antithrombin III (Hatton et al., 1978; Chan & Chan, 1979). Some authors claim that heparin is present in the vascular wall (Isuku & Murata, 1973). Moreover, the endothelium has been demonstrated to potentiate the action of antithrombin in a heparin-like way (Dryjski et al., 1983; Swendenborg et al., 1983) and heparin has also an antimitogenic effect, inhibiting smooth muscle cell growth and the development of neointimal fibrous hyperplasia (Karnovsky, 1981). These findings make it logical to attempt to bind heparin to vascular graft surfaces in order to achieve an antithrombotic effect.

Already in 1963, Gott et al. reported on a graphite-benzal-conium-heparin coating of plastic materials which resulted in reduced thrombogenicity at least in vitro. This surface exhibited net surface charges comparable to those of normal vessels. However, later studies of a similar surface showed rapid loss of heparin, the thrombus-inhibiting effect being only of brief duration (Kramer et al., 1967). In 1967, Eriksson et al. proposed a method of preparing a heparinized surface by ionic bonding. Since the stability of the heparin complex in contact with blood turned out to be unsatisfactory, the surface was later modified and stabilized with glutaraldehyde treatment (Lagergren & Eriksson, 1971). This surface has been further developed (Olsson et al., 1977; Larsson, 1980). Later studies demonstrated that plastic surfaces coated with different glucosaminoglycans were platelet compatible (Larsson et al., 1979). In addition, it was demonstrated that a heparin coated surface has the ability to inhibit adsorbed thrombin, and thus acts like an immobilized anticoagulant (Larsson et al., 1980). Later a technique to bind heparin covalently via a terminal residue was developed; this gives a stabilized bonding with more active sites available than with conventional covalent bonding methods (Larm et al., 1983). The latter two bonding methods have been applied in the present study to vascular graft surfaces.

Additional scattered reports on heparin bonding to artificial surfaces have appeared in the literature (Chawla & Chang, 1974; Lindsay et al., 1976; Ebert & Kim, 1982; Mangano, 1982). In a recent review Hufnagel summarized that no satisfactory solutions have yet evolved (1982). He presented results of heparin bonding to the modified human umbilical vein by simple incubation, demonstrating that surface concentrations of heparin were more dependent on the concentration of heparin in the solution than on the exposure time. The half-life of such bonding seemed to be limited to a few days, but even this was considered of value in preventing early clot formation. The results of his experiments

indicated the presence of binding sites for heparin also on vessels treated with glutaraldehyde. Preliminary results of incorporation of heparin into PTFE grafts indicated the possibility of a similar phenomenon with this synthetic surface.

OTHER METHODS TO IMPROVE GRAFT PERFORMANCE

Systemic antithrombotic therapy

Since the introduction of heparin in vascular surgery in 1940 (Murray, 1940) its use was long considered mandatory (Barner, 1974). However, heparin therapy is not only associated with risk for bleeding complications, but thrombo-embolic complications may also be related to the administration of heparin (Baird & Convery, 1977).

During the last decade different platelet function inhibitors have increasingly been used to reduce postoperative graft thrombosis, and several authors have reported experimental (Oblath et al., 1978b; Feins et al., 1979; Hagen et al., 1982) and clinical (Harjola et al., 1981; Green et al., 1982; Goldman et al., 1983a) success using aspirin and dipyridamole.

The somewhat confusing literature on medical prevention of occlusion after arterial reconstructions has recently been reviewed by Bergqvist (1983). He concluded that dextran or heparin should be used intraoperatively and that there is evidence suggesting a beneficial antithrombotic effect of the combination of ASA and dipyridamole in peripheral reconstructive surgery, and thus this treatment was recommended.

Like the group of non-steroidal anti-inflammatory agents, aspirin is an inhibitor of cyclo-oxygenase and thus also blocks the synthesis of prostacyclin (see page 12!). An intense research has been carried out in order to identify substances with a specific effect on thromboxane formation. Ibuprofen blocks cyclooxygenase, but has also been claimed

to inhibit thromboxane specifically (Carson et al., 1981). It was demonstrated to inhibit arterial graft thrombosis in a sheep model (Carson et al., 1981), and Claus et al. (1982) demonstrated improved patency of PTFE microarterial prostheses. Prostacyclin is theoretically the ideal antiaggregatory substance. However, it also acts vasodilating, which may result in hypotension. For practical reasons such as its instability and cost, it is not a realistic alternative. Neither are any synthetic analogues as yet clinically available. During the last few years, a number of substances with specific inhibitory effects on thromboxane synthesis have been produced. One of these, the imidazole derivative Dazoxiben, has been studied regarding its effects in vitro and in vivo both in animals and humans (Randall et al., 1981; Randall & Wilding, 1982), as well as experimentally regarding its ability to inhibit arterial graft thrombosis (Goldman et al., 1983d; Hanson & Harker, 1983).

THE PRESENT INVESTIGATION

The ideal vascular prosthesis with properties resembling those of the normal human artery has not yet been developed. However, for reasons stated above, there exists a great need for a small arterial substitute at least comparable to the autologous saphenous vein. Vollmar (1980) classified graft failures as acute, early or late, the acute failures occurring during the first three days postoperatively. In analogy to this, thrombogenicity can be graded as acute or chronic. The present investigation focuses mainly on the acute thrombogenicity, in vivo studies evaluating the thrombotic events occurring during the first 3-4 hours of blood/surface contact.

AIMS

- To evaluate in vitro the capacity of modified human umbilical vein (Biograft) and polytetrafluoroethylene (PTFE) to adsorb and inactivate thrombin and to compare the results to those obtained for native vessels (I-III).
- To study in vitro the effect on the thrombin inhibiting capacity of different methods of heparin treatment of graft surfaces (I-III) and also to compare the effect of conventional and low molecular weight (LMW) heparin bound to the surface of Biografts (II).
- To evaluate in vivo the acute thrombogenicity of Biograft, PTFE and polyurethane (PU) in low flow conditions, to assess the effect of heparin treatment of the surfaces (IV-VI), and to compare the results to those obtained for autologous arteries.
- To study ex vivo the production of prostacyclin and thromboxane in Biografts implanted for different lengths of periods (VII), and to evaluate the effect on the acute thrombogenicity of systemic treatment with a thromboxane synthesis inhibitor (VIII).

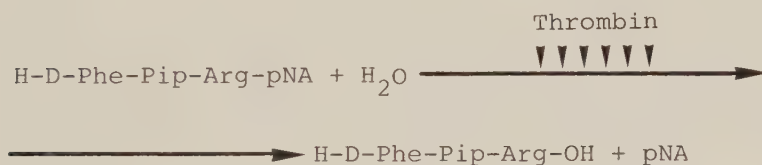
METHODS AND MATERIALS

For detailed information, reference is made to the individual papers (I-VIII)

IN VITRO UPTAKE AND INHIBITION OF THROMBIN (I-III)

This method was developed by Larsson et al. (1980) and utilizes a chromogenic peptide substrate to assess thrombin concentrations on surfaces and in solutions.

Chromogenic substrates are small peptides, which mimic natural substrates for which the primary structure around the cleavage site is known. The structure of the substrate preceding the bond split by the enzyme is imitated and to this peptide structure a chromophore - paranitroanilide (pNA) - is coupled. Enzymatic activity releases pNA, the determination of activity being based on the difference in optical density (absorbance) between the pNA formed and the original substrate (Fig. 2).



pNA (paranitroanilide) is measured spectrophotometrically at 405 nm.

Fig. 2. Principle for substrate S-2238 for thrombin.

Substrate S-2238 used in this study has a high specificity for thrombin, the rate of hydrolysis caused by other plasma proteolytic enzymes being less than 2% of the rate caused by thrombin comparing equimolar amounts of the enzyme (Claesson

et al., 1979). The sensitivity of S-2238 for thrombin is even higher than that of the natural substrate fibrinogen (Claesson et al., 1979).

The experimental procedure is outlined in Fig. 3. The thrombin concentration in the solution was assayed before and after exposure to the surfaces by use of the chromogenic substrate and the loss calculated (A). The surfaces were then exposed to the substrate before and after incubation with antithrombin III (B and C) and the ability of the surface to enhance the inhibition of thrombin evaluated.

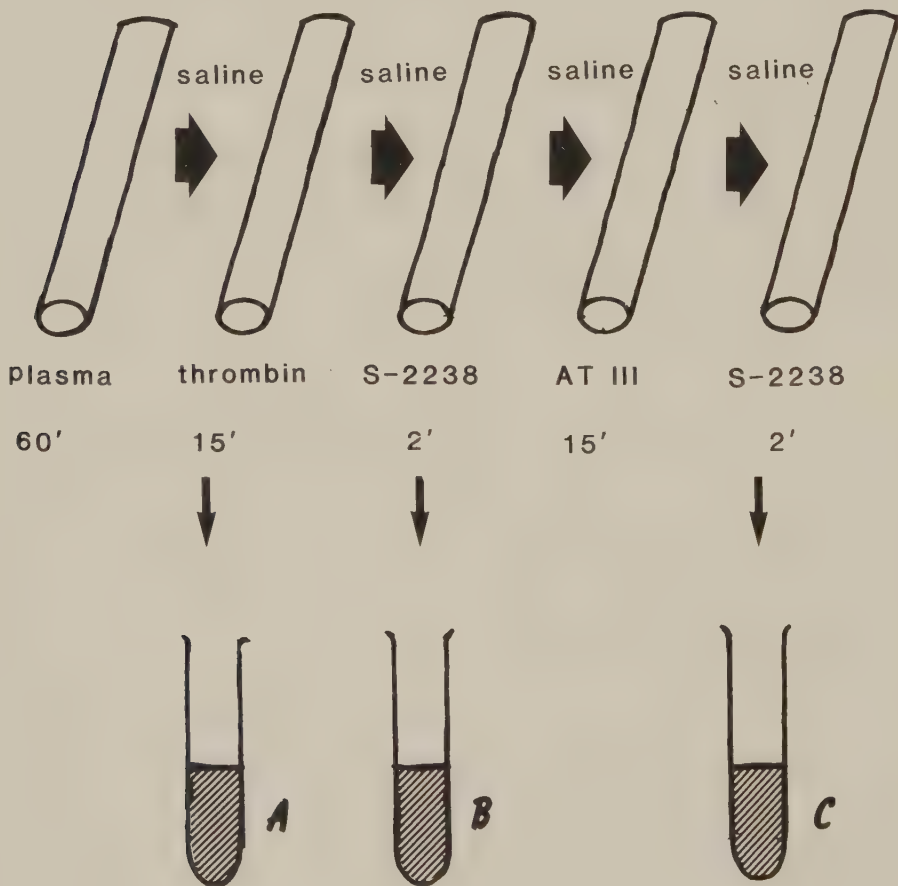


Fig. 3. Experimental procedure.

Effect of protamine on the different surfaces was studied by incubating them with protamine chloride prior to exposure to the thrombin solution.

Comments

Surface confined thrombin activities were expressed as estimated NIH Units/cm² surface area. These values are based on a standard curve for thrombin in solution and are not comparable to NIH Units of thrombin in solution. Thus, values for surface confined thrombin concentrations expressed as estimated NIH Units/cm² may be larger than those for loss from the solutions.

Conventional heparin is a large and heterogenous substance regarding molecular weight, chemical composition and effects on the hemostatic system (Jaques & Kavanaugh, 1973; Andersson et al., 1976; Laurent et al., 1978; Holmer et al., 1981). Heparin fragments of low molecular weights strongly potentiate inhibition of Factor Xa, whereas they do not appear to affect thrombin inhibition significantly or to prolong the activated partial thromboplastin time (Holmer et al., 1980). From a theoretical point of view, it would therefore be possible to prevent thrombosis with less risk for bleeding complications, which could be an advantage in vascular surgery. Certain low molecular weight (LMW) heparin preparations have been shown to prevent experimental venous thrombosis (Holmer et al., 1982; Björck et al., 1984), and, in addition, they do not influence haemostatic plug formation, as conventional heparin does (Esquivel et al., 1982). Carter et al. (1982) demonstrated that heparin depolymerized by nitrous acid degradation to LMW fragments showed greater antithrombotic activity than standard heparin in rabbits. The fragments also caused less blood loss.

A high affinity for antithrombin, necessary for the anticoagulant effect of a heparin fragment, is also important for the effects on platelets, fragments with high affinity for

antithrombin III having the least platelet activating effect (Holmer et al., 1980; Salzman et al., 1980). It has therefore been suggested that, when heparinizing artificial surfaces, low molecular weight heparin with high affinity for antithrombin should be used to achieve improved thromboresistance (Salzman et al., 1981).

The LMW heparin fragment used in the present study was obtained by nitrous acid degradation of standard porcine mucosal heparin and has a high affinity for antithrombin III and an antifactor Xa/APTT ratio of 4,1:1 (Holmer et al., 1983).

Protamine has been used as the principal agent for neutralizing heparin. It acts by dissociating the heparin-AT III complex by means of binding to heparin (Okajima et al., 1981). In vitro, this inactivation has been demonstrated to be dependent on the molecular weight of the heparin preparation. Thus, the anti-Xa activity of standard heparin was inhibited in parallel with the APTT activity, but only 25% of the anti-Xa activity of the fragment was neutralized at a concentration abolishing its APTT activity (Holmer & Söderström, 1983).

IN VIVO ACUTE GRAFT MODELS (IV-VI, VIII)

Vascular prostheses were anastomosed to the carotid arteries of dogs or sheep. In the first experiments systemic heparinization was used during the insertion of the grafts, and the heparin effect was reversed by protamine (IV). Since the effects of protamine on surface-bound heparin are unclear (see Papers II-III), the remainder of the experiments were performed using heparin solution (4 IU/ml) to flush the transected vessels, and thus no protamine was needed (V, VI, VIII).

Grafts were perfused for 3 or 4 hours. Blood flow through the grafts was reduced in order to accelerate the thrombotic process, by placing a special clamp distally according to

Sauvage et al. (1979). Autologous platelets were labelled with ^{51}Cr or ^{32}P , the activity being recorded at regular intervals during the whole perfusion period using an external detector. The activity for ^{51}Cr was correlated to the activity in whole blood, whereas the ^{32}P -activity was determined as the percentage increase in the initial activity at a reference point on the artery proximal to the graft. In addition, the ^{32}P activity in platelet-rich plasma was determined. In the first experimental set-up ^{125}I -fibrinogen accumulation in the grafts was also measured.

Blood flow was checked by an electromagnetic flowmeter, which was also used to determine patency. After excising the grafts, they were opened longitudinally, thrombotic material removed and weighed and luminal surfaces photographed for determination of thrombus free surface (TFS).

Comments

Type of animal. The most popular animals for the study of vascular prostheses are dogs and baboons. While baboons have the advantage of being hematologically closer to humans (Harker et al., 1979), dogs differ in blood coagulation, fibrinolysis and platelet function. For practical reasons baboons cannot be utilized at our laboratory, and since dogs are frequently used at several other laboratories, the first study was made using dogs (IV). Other animals available for the study of vascular grafts are pigs and sheep. Sheep have a coagulation system resembling the human (Tillman et al., 1981) and certain other advantages such as low cost, long and accessible carotid arteries and easy handling. Sheep were thus used in studies V, VI, and VIII.

Type of isotope. ^{51}Cr has been the isotope most frequently used for labelling platelets both for studies of platelet survival and turnover (Ljungqvist, 1970) and platelet accumulation on artificial surfaces (Hanson et al., 1980; Roedersheimer et al., 1980). The labelling technique

was described by Aas and Gardner (1958) and modified by Abrahamsen (1968), resulting in increased circulating platelet activity. However, ^{51}Cr is a gamma-emitter, the activity of which is difficult to isolate at the level of a vascular graft, since the activity from the remainder of the animal has to be shielded. In the experiments using this isotope (IV), isolation of the grafts was accomplished by using long graft segments (20 cm), which were exteriorized and placed in circular lead shields in a loop fashion.

^{32}P is a beta-emitter with short-range radiation. This made it possible to use shorter graft segments (6 cm) which could be placed in rather thin aluminium shields, preserving the anatomical position of the vessels. Other problems such as turbulence and kinking of the grafts were thus avoided.

Labelling was performed using ^{32}P -orthophosphate in HCl (pH 2-3) and an incubation period of 2 hours as described by Wieslander (1982). The ^{32}P -orthophosphate has been demonstrated to become incorporated into platelet phospholipids both in vitro and in vivo (Leung et al., 1977). In addition, it has been shown that labelling with ^{32}P does not damage the platelets and that the release reaction is minimal (Ebbe, 1965). It has also been demonstrated, in the rabbit, that the platelet activity follows the whole blood activity (Ebbe, 1965). The use of ^{32}P also allowed the regional distribution of platelet deposition along the length of the graft to be studied.

Flow reduction. In order to accelerate the thrombotic process, the flow through the grafts was reduced. This was accomplished by placing a special clamp distal to the graft. In the first experiments flow was restricted to 50 ml/min (IV) and in the rest the flow was 25 ml/min.

Patency, TFS and thrombus weight. A graft may be thrombogenic and yet remain patent during an acute experiment. Thus, a visual inspection of the graft surface may add

valuable information, even though the TFS estimate is to some extent subjective and does not give information about the composition of the thrombotic material. However, by evaluating TFS, weight of thrombus and the macroscopic appearance of the surface, substantial information may be gained.

HEPARIN TREATMENT OF GRAFT SURFACES

Four types of heparin treatment were applied to the surfaces. For detailed description see the individual papers.

A. Heparin incubation of Biografts (I,IV)

Biografts were incubated in a commercially available heparin solution for at least 12 hours (I), or flushed with saline containing heparin according to the prescriptions of the manufacturer (IV).

B. Heparin and alcohol treatment of Biografts (I,II,IV,V)

Following heparin incubation as in A, grafts were stored in 50% ethylalcohol.

C. Stabilized ionic heparin bonding to synthetic (PTFE and PU) grafts (VI)

Grafts were supplied with a glutaraldehyde-stabilized heparin layer according to a method described and evaluated elsewhere (Olsson, 1977; Larsson, 1980).

D. Covalent heparin bonding to Biografts and PTFE grafts (II,III,V,VI)

This method is similar to the ionic bonding method and has been described in detail elsewhere (Larm et al., 1983).

Comments

The prescriptions for preparation of Biografts prior to implantation include flushing with saline containing 10000 IU/l of heparin and as a final step flushing with 30000 IU of heparin (water solution 5000 IU/ml) (Dresner & Turner, 1980). As stated earlier Hufnagel (1982) has demonstrated the amount of heparin bonded to Biograft surfaces to be more dependent on the concentration of the heparin solution used than on the exposure time. Thus, he found that heparin concentrations of 5000 IU/ml or more resulted in considerable amounts of surface-bound heparin. Using various incubation times he showed that incubation periods longer than 15 minutes gave only marginal increases in the surface concentration of heparin. Glimelius et al. (1978), studying heparin binding to cultured endothelial cells, demonstrated an increase in surface bound heparin only during the first 2-4 hours. In the present investigation, we used 12-24 hour incubation time and a concentration of 5 000 IU/ml, and this may be considered sufficient according to the findings mentioned above. In the in vitro investigation (I), both groups of heparin treated Biografts were incubated in heparin for at least 12 hours, but in the in vivo experiments studying the effect of alcohol on the heparin layer (IV), the "non-alcohol" group was treated according to the prescriptions of the manufacturer (IV), i.e., the exposure time for heparin was considerably shorter. However, there was no difference with amount of labelled heparin on the surfaces between heparin incubated and heparin flushed grafts (Esquivel, 1982).

The stabilized ionic and covalent bonding methods, were performed by IRD Biomaterial AB, Bromma, Sweden. These methods include several steps, some of which are performed at temperatures above the body temperature (50⁰ C). They have been extensively described and studied elsewhere (Olsson et al., 1977; Larm et al., 1983).

PRODUCTION OF THROMBOXANE/PROSTACYCLIN (VII, VIII)

The production of thromboxane and prostacyclin from specimens taken from both anastomotic and midgraft regions was determined. Sections from normal arteries and veins were used as controls. Commercial radioimmunoassays (RIAs) using labelled antigen and antirabbit antiserum as antibodies for TxB_2 and 6-keto-PGF $_{1\alpha}$ were used.

Comments

Thromboxane A_2 (TxA_2) and prostacyclin (PGI_2), two of the biologically active products of prostaglandin endoperoxide metabolism, are very unstable in the circulation. The stable end products of TxA_2 and PGI_2 , thromboxane B_2 (TxB_2) and 6-keto-PGF $_{1\alpha}$ respectively, can be assayed using RIA kits. According to determinations made by the manufacturer the antibodies of these cross-react only to a small degree with other prostaglandin endoperoxides. The results were correlated to those for normal sheep arteries and veins.

ASSESSMENT OF ANTITHROMBOTIC EFFECTS OF INHIBITION OF THROMBOXANE SYNTHESIS (VIII)

In addition to its effect on arterial graft thrombosis, the effects of Dazoxiben on microvascular hemostasis, platelet activity following laser injury and experimental venous thrombosis formation were studied.

Acute arterial graft thrombogenicity. Non-heparinized Bio-grafts were studied using the same experimental model as in papers V and VI in control animals and animals receiving Dazoxiben i.v.

Microvascular hemostasis. Hemostatic plug formation in arterioles and venules of the rabbit mesentery was studied according to the technique described by Bergqvist (1973).

Platelet activity following laser injury. Microarterial embolism following a laser injury to arterioles was studied intravitaly. The technique has previously been described by Arfors et al.(1970).

Venous thrombosis formation. Venous thrombosis was induced according to Peterson and Zucker (1970) as modified at our laboratory (Björck et al., 1984) by a combination of endothelial damage and flow reduction.

Comments

The objective of this study was to evaluate the effect of Dazoxiben on acute arterial graft thrombosis, to study changes in the production of prostaglandin metabolites and to assess the effect of Dazoxiben on venous thrombosis and on platelets in two other models, laser induced microembolism and hemostatic plug formation. Experimental venous thrombosis formation in the rabbit can be counteracted by heparin and heparin analogues (Björck et al.,1984) and dextran, which affects platelets and blood flow (Ah-See et al., 1974), but not by different platelet function inhibitors (Arfors et al., 1975). These studies indicate that platelet activation has a relatively small influence on the initiation of venous thrombosis in this model, whereas diminished flow and coagulation activation are important factors.

Both laser induced embolism and hemostatic platelet plug formation are primarily due to ADP-induced platelet aggregation, collagen probably being of minor importance (Hovig et al., 1974; McKenzie et al., 1974; Born et al., 1976; Bergqvist & Arfors, 1980).

EXPERIMENTAL DESIGN

The designs of the various parts of this study are outlined in Tables I-III.

IN VITRO STUDIES (I-III)

TABLE I. Surfaces studied in the different papers regarding:
A/ Uptake and inhibition of thrombin.
B/ Effect of protamine.

Surface	A Paper no.	B Paper no.
Fresh human umbilical vein	I, II	II
Non-heparinized Biograft	I, II	--
Saline-alcohol Biograft	II	--
Heparin incubated Biograft	I	--
Heparin-alcohol Biograft	I, II	II
Covalently-heparinized Biograft	II	--
LMW heparin-alcohol Biograft	II	II
Non-heparinized PTFE	III	--
Covalently-heparinized PTFE	III	III
Sheep aorta	III	--
Sheep carotid artery	III	--
Sheep jugular vein	III	--

IN VIVO ACUTE GRAFT MODELS (IV-VI, VIII)

TABLE II. Surfaces studied in the different papers with reference to:

A/ Type of animal.

B/ Graft length (cm).

C/ Type of isotope used to label platelets.

D/ Flow (ml/min).

E/ Length of perfusion period (minutes).

Surfaces studied	A	B	C	D	E
Paper IV, <u>Biograft</u>					
Non-heparinized					
Heparin-flushed	Dog	20	⁵¹ Cr	50	180
Heparin-alcohol					
Paper V, <u>Biograft</u>					
Non-heparinized					
Heparin-alcohol	Sheep	6	³² P	25	240
Covalently-heparin.					
Autologous artery					
Paper VI, <u>PTFE</u>					
Non-heparinized					
Ionic heparinized					
Covalently-heparin.	Sheep	6	³² P	25	180
<u>Polyurethane</u>					
Non-heparinized					
Ionic-heparinized					
Paper VIII					
Biograft (Non-heparin)	Sheep	6	³² P	25	240

Comments (Table II). Grafts were evaluated in vivo as to their patency and accumulation of labelled platelets, and ex vivo concerning thrombus free surface and thrombus weight. In paper IV ¹²⁵I-labelled fibrinogen was also used.

PRODUCTION OF THROMBOXANE AND PROSTACYCLIN (VII-VIII)

TABLE III. Production of thromboxane and prostacyclin in the pseudointima of Biograft. Length of implantation period, flow, thromboxane synthesis inhibition with Dazoxiben.

Paper	Length of implantation	Flow	Dazoxiben
VII	10 days	50 ml/min	No
VII	90 days	"physiologic"	No
VIII	4 hours	25 ml/min	Yes

Heparin incubated and heparin-alcohol treated Biografts were used in paper VII. Since no differences were noted between the two graft types the data are presented as one group. All grafts in paper VIII were non-heparinized Biografts. One group of animals received Dazoxiben, the other animals received no medication, serving as controls. Paper VIII also includes studies in rabbits of haemostatic plug formation time, laser-induced microembolism and experimental venous thrombosis formation. In the first two models determinations were made before and after administering Dazoxiben and each animal thus served as its own control. In the venous thrombosis model, three groups were studied: one control group receiving saline, and two treatment groups receiving 10 and 25 mg/kg Dazoxiben respectively. Sections for determination of thromboxane/prostacyclin production were taken from injured and non-injured veins in the venous thrombosis experiments.

STATISTICAL METHODS

Information on the statistical methods is given in the individual papers.

RESULTS

IN VITRO STUDIES

Paper I. Native umbilical veins were found to possess the ability to adsorb and inactivate thrombin on contact with antithrombin III. This property was lost after treating the veins with glutaraldehyde to produce vascular grafts (non-heparinized Biografts) but was restored after heparin-alcohol treatment. The group of grafts incubated only in heparin showed an insignificant thrombin-inhibiting capacity, but only consisted of 4 determinations. However, later experiments confirmed these findings, heparin incubated grafts behaving in a very irregular fashion (0.26 ± 0.32 NIH Units/cm² of surface bound thrombin, residual thrombin after AT III exposure $47 \pm 31\%$, $n = 7$ and $n = 3$, respectively, unpublished data). This phenomenon could probably be explained by an instability of the heparin layer.

Paper II confirmed the findings of paper I concerning non-heparinized and heparin-alcohol treated Biografts. In addition, it was demonstrated that Biografts treated with saline and alcohol behaved in the same way as non-heparinized grafts, concerning both adsorption and inactivation of thrombin (residual thrombin after AT III $79 \pm 17\%$ and $77 \pm 19\%$ for non-heparinized and saline-alcohol Biografts respectively).

Comparing LMW heparin-alcohol and conventional heparin-alcohol Biografts, the former adsorbed more thrombin and exhibited a less pronounced ability to inactivate thrombin (residual thrombin after AT III 30 ± 12 and $7 \pm 10\%$ for LMW heparin-alcohol and heparin-alcohol surfaces respectively). The covalently-heparinized surface showed values comparable to those for the LMW heparin-alcohol grafts, but in the former group only two grafts were available for study.

Protamine exposure of the various surfaces resulted in a

greater uptake of thrombin on heparin-alcohol surfaces ($p < 0.025$) and an impaired ability to inactivate thrombin ($p < 0.005$). On the other hand, LMW heparin-alcohol surfaces showed a decreased uptake of thrombin ($p < 0.005$), but their thrombin inhibiting ability was totally abolished ($p < 0.005$). In fact, this surface did not differ from the non-heparinized one. The native umbilical vein was not affected by exposure to protamine, concerning either loss from the solution, or its capacity to inactivate thrombin.

Paper III. Untreated PTFE did not adsorb thrombin to any significant degree, heparin treatment of this surface resulting in a pronounced ability to adsorb and inactivate thrombin. This surface exhibited the highest value for thrombin adsorption (1.13 ± 0.17 NIH U/cm²), residual thrombin after AT III exposure being only $23 \pm 10\%$.

Both sheep veins and arteries also showed excellent thrombin inhibiting ability, but comparing loss from the thrombin solution and the subsequent adsorption on the surfaces, there was a marked difference between those types of vessels. While losses from the solution were comparable, the amount of surface confined thrombin was significantly lower on arteries than on veins ($p < 0.005$). Protamine treatment of covalently-heparinized PTFE resulted in strongly decreased figures both of loss of thrombin from the solution and of adsorption on the surface ($p < 0.005$).

IN VIVO ACUTE GRAFT MODELS

Paper IV. Non-heparinized Biografts showed the highest thrombogenicity, as evaluated by patency, TFS, thrombus weight and platelet accumulation in the grafts. Both heparin flushed and heparin-alcohol Biografts showed 100% patency, but the latter group of grafts exhibited the smallest platelet deposition and also had the largest TFS ($82 \pm 5\%$). There were no differences between surfaces studied regarding the amount of ¹²⁵I-fibrinogen deposition.

Paper V. As expected, autologous arteries showed excellent results with 100% patency and a minimal amount of thrombotic material. However, these grafts exhibited an obvious increase in radioactivity during the first part of the perfusion period. The activity decreased continuously towards the ends of the experiments, values at 240 minutes approaching these found at the reference point (Fig. 4).

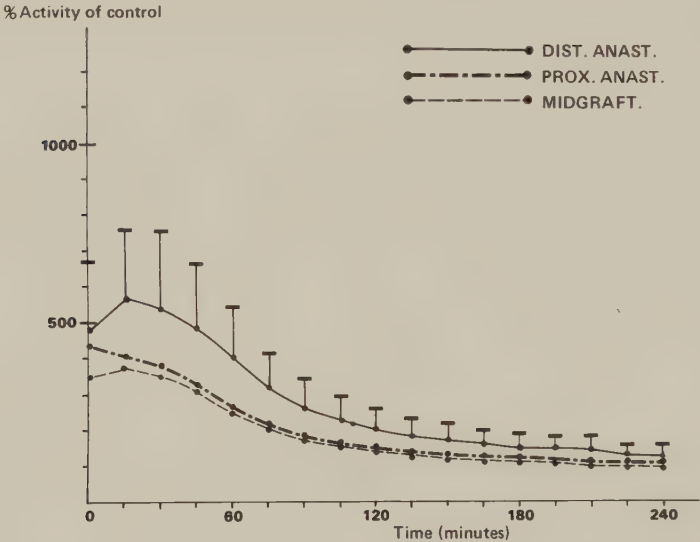


Fig. 4. ^{32}P -platelet accumulation in the different regions of autologous arteries ($n=9$; M, SEM is indicated for the distal anastomosis).

The low flow of these experiments resulted in a non-heparinized Biograft occlusion frequency of almost 100% (11/12), whereas only 2 of 8 heparin-alcohol treated grafts occluded ($p<0.01$). Thrombi were also significantly smaller in heparin-alcohol treated grafts ($p<0.01$). As compared with non-heparinized grafts platelet accumulation was significantly decreased in heparin-alcohol grafts at all positions, whereas covalently-heparinized Biografts showed an insignificant decrease. In addition, covalently-heparinized Biografts failed to distinguish themselves from non-heparinized grafts concerning also thrombus weight and patency.

Paper VI. Stabilized ionic bonding of heparin diminished the thrombogenicity of polyurethane (PU) but not of PTFE grafts. The TFS of PU grafts was significantly increased by heparinization ($p < 0.02$) and platelet accumulation was also significantly reduced in heparinized grafts compared to non-heparinized PU. In contrast to the findings with the ionic bonding to PTFE grafts, the covalently-heparinized PTFE grafts exhibited a reduced acute thrombogenicity, figures for TFS and platelet deposition being significantly lower than those for non-heparinized PTFE. The spatial distribution of platelets in PTFE grafts was, as in PU grafts, uneven, distal anastomoses exhibiting the highest activity.

PRODUCTION OF THROMBOXANE AND PROSTACYCLIN IN BIOGRAFTS

Paper VII. At both 10 and 90 days grafts produced more TxB_2 than carotid arteries, but no difference in the production of 6-keto-PGF_{1 α} was noted between grafts and carotid arteries. This resulted in a strongly decreased ratio of 6-keto-PGF_{1 α} / TxB_2 ($p < 0.05$).

Paper VIII. After the 4 hour perfusion period grafts implanted in sheep produced significant amounts of TxB_2 and 6-keto-PGF_{1 α} . This finding was related both to anastomotic and midgraft regions. Rabbit veins injured with ethoxysclerol showed an increased production of TxB_2 compared to noninjured veins from the same animal. There was a tendency towards decreased production of 6-keto-PGF_{1 α} in injured vessels.

EFFECTS OF INHIBITION OF THROMBOXANE SYNTHESIS

Paper VIII. Dazoxiben treatment failed to affect both hemostatic plug formation time and microvascular arteriolar embolism in rabbits. The frequency of jugular vein thrombosis was not decreased, but there was a decrease in thrombus weights at a dose of 10 mg/kg b.w. ($p < 0.05$). This was not observed in rabbits receiving a dose of 25 mg/kg b.w.

In untreated sheep only one of 12 Biografts remained patent, whereas the patency for Dazoxiben treated animals was 75% (6/8; $p < 0.01$). Thrombi were also significantly smaller in dazoxiben treated sheep ($p < 0.01$).

The reduced thrombogenicity of grafts in Dazoxiben treated animals was accompanied by a significant decrease in the production of TxB_2 from all regions of the grafts. A slight, but not significant, increase in 6-keto-PGF_{1 α} production was also encountered in the treatment group. Histologically scattered monocytes, but no platelets, were found on the graft surfaces. Rabbits treated with Dazoxiben showed a decreased production of TxB_2 in injured vessels down to the level encountered in non-injured vessels.

GENERAL DISCUSSION

The "ideal" vascular graft

The ideal substitute in vascular surgery has an intima with normal functions and a permanent elasticity, i.e. in essence it resembles the normal human artery. The latter is, however, a very complex organ, each component of which actively responds to the physiologic demands being placed upon it. These do not include only the transport of blood and maintaining the blood in the liquid state. The arterial wall has also to store potential energy, to maintain the variable tension required for distributing blood according to regional needs, to supply nutrition for parietal components and repair damage to its own wall. So far no graft material, except the autologous artery, meets all these demands. In addition, no characterization of a particular graft can be claimed to give a complete assessment of its functional properties or to give a reliable prediction of its long-term performance.

The methods of evaluating surface thrombogenicity, both *in vitro* and *in vivo* presented in this study, focus mainly on two particular characteristics: a) the ability of the surface to interact with coagulation enzymes, measured as its capacity to adsorb and inactivate thrombin (*in vitro*) and b) its tendency to accumulate thrombotic material as evaluated using radioactively labelled platelets and fibrinogen (*in vivo*).

In vitro methods

The native vessel wall has been demonstrated to possess specific binding sites for thrombin (Awbrey et al., 1979). This property may contribute to thrombo-resistance, since it has been demonstrated that circulating thrombin can thus be removed (Lollar & Owen, 1980). Surface-located glucosaminoglycans (GAGs) represent possible binding sites for thrombin

(Busch & Owen, 1982), and it has also been demonstrated that artificial surfaces with immobilized GAGs bind thrombin (Larsson et al., 1980). Surface-adsorbed thrombin is slowly inactivated by the endothelium, this process proceeding at a much faster rate on contact with plasma. Antithrombin III must be present for this to occur, although an additional, as yet unidentified, plasma factor seems to be required for instantaneous inhibition (Dryjski, 1984). So far the only artificial GAG surface, capable of inactivating adsorbed thrombin is one coated with heparin (Larsson et al., 1980).

Using an antithrombin III solution, the present study shows that the native umbilical vein endothelium possesses this ability to inactivate thrombin. The glutaraldehyde treatment applied in making Biografts destroys this capacity. This surface can thus be predicted as being thrombogenic, since it retains, or even increases its ability to adsorb thrombin. However, following heparin exposure, which is mandatory according to the prescriptions for using Biografts, the thrombin inhibiting capacity is restored. This is even more pronounced when the heparin incubation is followed by exposure to alcohol. PTFE surfaces lack the ability to adsorb significant amounts of thrombin. The predictive value of this finding is probably small, since other synthetic surfaces such as polyethylene (Larsson et al., 1980) and polyurethane (unpublished observations) also lack the capacity to adsorb thrombin. At least polyethylene is highly thrombogenic. However, when supplied with a stabilized heparin layer, PTFE surfaces not only adsorb considerable amounts of thrombin, but also develop an excellent ability to inactivate it.

Comparing results for heparinized graft surfaces to those obtained for native sheep vessels, it is obvious that heparin treatment of graft surfaces adds a property similar to that of normal vessels, as regards uptake and inhibition of thrombin. The results for sheep vessels indicate that veins and arteries differ in this respect, arteries inactivating

thrombin also in the absence of plasma. A possible explanation is the reported presence of AT III in the vessel wall (Chan & Chan, 1979).

Low molecular weight (LMW) heparin is a more attractive alternative than conventional heparin to use in connection with surgery because of a decreased influence on initial hemostasis (Esquivel et al., 1982; Carter et al., 1983). The present study shows that despite its weaker antithrombin capacitating ability it is also a realistic alternative for treatment of graft surfaces. However, the LMW heparin surface showed to be more susceptible to protamine. In fact, its thrombin inhibiting capacity, as compared to the non-heparin surface, was totally destroyed after protamine exposure. Surfaces with conventional heparin partly lost their initial capacity following exposure to protamine, as did covalently heparinized PTFE. It can thus be stated that protamine is also capable of inactivating surface-located heparin. However, the clinical relevance of this is uncertain, since doses of systemically administered protamine are adjusted to inactivate previously injected, circulating heparin, and thus protamine concentrations in vivo will not reach those used in the present in vitro experiments.

In vivo methods

The carotid artery implantation model advocated by Sauvage (1979) using controlled low flows, has been shown to be of predictive value regarding the long-term performance of a particular graft material and has also been used to evaluate the effect of platelet function inhibition (Sauvage et al., 1981; Carson et al., 1981). Using this model in dogs with exteriorized grafts, measured values of the accumulations of ^{51}Cr -labelled platelets and ^{125}I -labelled fibrinogen, confirmed the findings of the *in vitro* experiments. Not only was the usefulness of heparin incubation demonstrated, but the necessity of exposing the heparin treated surface to alcohol in order to increase the antithrombotic effect was

also confirmed. The effect of the alcohol incubation is unclear, but may perhaps be explained in terms of stabilization.

Modifications of the *in vivo* model, including the use of sheep instead of dogs, ^{32}P -labelling and registration of platelets as described by Wieslander et al. (1982), and restricting the flow to 25 instead of 50 ml/min, confirmed the results of the earlier experiments and, in addition, demonstrated the superiority of the autologous artery. This was also in accordance with *in vitro* results, showing that arteries have a higher antithrombotic potential than both veins and artificial surfaces. The autologous artery thus appears to be the ideal vascular substitute, an opinion previously expressed by others (Wylie, 1965).

Applied to the synthetic surfaces, polyurethane (PU) and PTFE, the stabilized heparin bonding methods, resulted in a significant reduction in thrombogenicity as measured by the amount of platelet accumulation in vivo (PU) and the gross accumulation of thrombotic material ex vivo (PU and PTFE). However, probably due to the surface characteristics of PTFE, the ionic bonding method was less suitable for this type of material. In addition, the covalent bonding method was not useful, when applied to the Biograft surface. This was not surprising since the graft is fragile and the various steps involved in the heparinization process probably result in extensive "intimal" damage, as was obvious from the visual inspection at removal of the grafts.

An almost constant finding as to the spatial distribution of platelets along the grafts, as recorded externally, was the higher accumulation at the distal anastomotic region, a finding reported previously also by others (Christenson et al., 1981b). Activation of platelets, when passing the proximal anastomosis and the foreign surface, may be an explanation of this phenomenon, other possible contributory factors being hemodynamic disturbances, i.e. turbulence.

Heparin desorption

Using labelled heparin Larsson et al. (1977) demonstrated that the glutaraldehyde-stabilized ionic heparin bonding to polyethylene surfaces was stable in vivo. The initial loss of activity was about 12%, whereafter no further loss was observed during the next seven days. This is probably also true for the covalent bonding method, many steps of which are similar to the ionic. By this method an ionic complex of heparin and hexadecylamine hydrochloride is stabilized on the surface with glutaraldehyde. The covalent bonding method comprises, as a last step, bonding of a partly degraded heparin, the molecule being immobilized via a terminal residue in a vertical position, thus leaving a larger number of active sites free to interact with AT III.

Using tritium-labelled heparin we have studied heparin desorption from Biografts incubated with heparin only, or with heparin and alcohol, after implantation periods of 10 and 90 days in sheep carotid arteries (Esquivel et al., 1983). Heparin-alcohol treated grafts retained significantly greater amounts of heparin after 90 days, the residual activity being about 40% for heparin-alcohol and <10% for heparin treated grafts respectively. The patency-rates for both types of grafts in this study were equal (1/6 thrombosed in each group) but with this low failure rate and low number of experiments a difference can hardly be expected.

Production of thromboxane and prostacyclin

The production of thromboxane A_2 and prostacyclin (PGI_2) as reflected by the amounts of their stable end products TxB_2 and 6-keto- $PGF_{1\alpha}$ eluted from graft specimens, was assessed by RIA-kits. The antibodies of these cross-react only to a very small degree with other prostaglandin endoperoxides, according to determinations made by the manufacturer. It is much discussed whether the amounts of these end-products actually reflect the total amounts of TxA_2

and PGI_2 since other metabolic pathways may be present (Kindahl & Granström, 1980). This is of less importance, however, since only relative values and differences as compared with native vessels were measured. It was demonstrated that the pseudointima produced an increased amount of thromboxane compared to carotid arteries and veins both after 10 and 90 days, whereas the production of 6-keto-PGF_{1 α} was not significantly altered. The resulting decrease in 6-keto-PGF_{1 α} /TxB₂ ratio indicates a low resistance to platelet aggregation, which may contribute to a low thromboresistance. Increased thromboxane production was also demonstrated in the acute experiments. The source of the thromboxane production in these experiments may be cells adhering to the graft surface. Not only platelets, but also other types of cells such as endothelial cells (Hopkins et al., 1978), leukocytes (Goldstein et al., 1978) and smooth muscle cells (Ody et al., 1982) do produce thromboxane. However, sheep platelets have been reported to produce only minute amounts of thromboxane (Hwang et al., 1980). The microscopic studies of grafts used in the acute experiments moreover demonstrated the presence of monocytes but not of platelets, and monocytes are known to contain large amounts of arachidonic acid (Nathan et al., 1980). It may therefore be suggested that monocytes are the possible origin for thromboxane.

The increased production of thromboxane from graft surfaces is probably a factor contributing to early graft failure. It is thus logical to try to inhibit the thromboxane synthesis pathway, by use of a drug with a specific activity on thromboxane synthetase.

Dazoxiben, an imidazole derivative, has been demonstrated to inhibit thromboxane synthesis both in vitro and in vivo (Randall, 1983), this being accompanied by an increase in prostacyclin levels. It probably acts by reorientating the arachidonic acid metabolism, and it has recently been demonstrated an increased production of other prostaglandin

endoperoxides, acting both pro- and anti-aggregatory (Gresele et al., 1983).

The present study shows that the concept of thromboxane synthesis inhibition is applicable in attempts to affect acute arterial graft thrombosis. Thus, Dazoxiben administered in relevant doses inhibited the production of thromboxane from graft surfaces, this being accompanied by a reduction in thrombogenicity as measured by in vivo determination of platelet accumulation, and ex vivo evaluation of TFS and thrombus weight. Patency was also significantly improved. These findings are contrary to those of Goldman et al. (1983d) who evaluated Dazoxiben in an artificial circulation simulating a femoropopliteal bypass and, despite a significant reduction in plasma levels of thromboxane, noted no effect on platelet deposition. In an in vivo arteriovenous shunt model in baboons Hanson & Harker (1983) also failed to demonstrate an effect on thrombosis formation. This difference may, at least in part, be due to the species difference. Different experimental conditions may be another explanation.

Our results also indicate that there is little influence on initial hemostasis by Dazoxiben. This is of no surprise since both laser-induced microarterial embolism and hemostatic plug formation are primarily caused by ADP-induced platelet aggregation (Hovig et al., 1974; McKenzie et al., 1974; Born et al., 1976; Bergqvist & Arfors, 1980), Dazoxiben only at very high concentrations influencing this (Heptinstall et al., 1980).

The failure of Dazoxiben to inhibit venous thrombosis formation, as induced in the present study, favours the opinion that platelet activation by arachidonic acid metabolites does not have a critical importance in the initiation of venous thrombosis (Schafer & Handin, 1979), and indicates that any future, clinical use will be restricted to the prevention of thrombotic events on the arterial side.

In summary, the methods used in the present study to evaluate the thrombogenicity in vitro and in vivo of various surfaces have given consistent results. Thus, surfaces which in vitro adsorb and fail to inactivate thrombin, show evidence of a high platelet accumulation in vivo and exhibit low patency-rates (non-heparinized Biograft). Surfaces which, on the other hand, possess in vitro the ability to adsorb and inactivate thrombin, perform significantly better than non-heparinized ones when implanted in vivo (heparin-alcohol Biograft, heparinized PTFE and autologous artery). Moreover, the pseudointima of Biografts has been demonstrated to produce increased amounts of thromboxane, which may contribute to the high thrombogenicity of these grafts. Selective inhibition of thromboxane synthesis with Dazoxiben, reducing the production of thromboxane, was accompanied by a significantly reduced acute thrombogenicity.

CONCLUSIONS

The present study allows the following conclusions:

- The autologous artery has excellent antithrombotic properties as evaluated by the present methods. This is consistent with the general opinion that autologous artery is the ideal vascular substitute.
- Non-heparinized Biograft has a high thrombogenicity, demonstrated as a low thrombin-inhibiting ability in vitro and a high tendency to accumulate platelets in vivo.
- Heparin and alcohol treatment of Biograft markedly reduces the acute thrombogenicity, as determined both in vitro and in vivo.
- Treatment of Biograft surfaces with low molecular weight (LMW) heparin and alcohol results in a significant ability of the surfaces to adsorb and inactivate thrombin. Although this property is more pronounced when conventional heparin is being used, LMW heparin appears to be a realistic alternative when "heparinizing" vascular graft surfaces.
- Stabilized heparin bonding reduces the acute thrombogenicity of synthetic grafts, as determined both in vitro (PTFE) and in vivo (PTFE and PU).
- The pseudointima or blood cells adhering to the inner surface of Biografts produce increased amounts of thromboxane as compared with normal arteries and veins.
- Inhibition of thromboxane synthesis reduces the acute thrombogenicity of Biografts.

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....., and finally, enjoy the communal feeling that perhaps, all of us together have already published Complete Truth, but that not a single one of us shall ever be able to read it.

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UPTAKE AND INACTIVATION OF THROMBIN BY THE FRESH, GLUTARDIALDEHYDE OR HEPARIN TREATED HUMAN UMBILICAL CORD VEIN ENDOTHELIUM.

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ABSTRACT With the aim to elucidate the non-thrombogenic properties of the vascular endothelium and the lining of a certain arterial substitute, the uptake and inactivation of thrombin by the native and modified human umbilical cord veins were investigated. The modification procedures included variations with glutardialdehyde treatment, heparin exposure and storage in ethyl alcohol. The native and all the modified veins adsorbed substantial amounts of thrombin, which was immobilized in an active state. Following exposure to antithrombin III solution, the adsorbed thrombin was inhibited only on the native veins and those treated with glutardialdehyde, stored in ethyl alcohol, exposed to heparin and again stored in ethyl alcohol.

INTRODUCTION

The healthy natural vascular wall appears to be the ultimate non-thrombogenic surface but its essential properties in this respect have remained elusive indicating a multifactorial complexity. A number of properties of the endothelial cells

KEY WORDS: Non-thrombogenic surface, mucopolysaccharides, thrombin, adsorption, inhibition, human umbilical cord vein

possibly related to their compatibility with the plasma coagulation system have been reported, such as the superficial presence of glucosaminoglycans (1,2) and antithrombin III (3), specific binding sites for heparin (4) and for thrombin (5) and secretion of substances affecting the fibrinolytic system (6). Platelet compatibility has been attributed to secretion of prostacyclin (7) from the endothelium.

In a recent study we found that a polyethylene surface modified to carry sulphate groups or coated with various glucosaminoglycans had the capacity to adsorb and immobilize thrombin in an enzymatically active state. Despite the fact that all the surfaces were platelet compatible only the heparin coating created a non-thrombogenic surface. This surface, in contrast to the other surfaces, had the regenerative capacity to inhibit the immobilized thrombin by an interaction with plasma or antithrombin III (8,9,10).

The present investigation was designed to elucidate the anticoagulant properties of the vascular endothelium. The glutardialdehyde tanned endothelial lining of the fresh human umbilical cord vein with and without subsequent heparin exposure and the native vein, were examined with regard to thrombin adsorption and inhibition together with antithrombin III.

MATERIALS AND METHODS

Glutardialdehyde, 25% (BDH Chemicals Ltd, England). An aqueous dilution at a concentration of 2% was used.

Human albumin, Kabi 20% (AB Kabi, Stockholm, Sweden). Diluted to 4% in physiological saline.

Thrombin, Topostasin Roche (Hoffman - La Roche, Basle, Switzerland). One ampoule (3000 NIH) was dissolved in 30 ml of distilled water and stored at -70°C . The activity given by the manufacturer was accepted for calculation of the final concentration. The stock thrombin solution was dissolved in the human albumin solution to a concentration of 20 NIH Units/ml. This solution was used in the experiments.

Heparin, 5000 IE/ml (Vitrum, Sweden).

Human antithrombin III solution, (AB Kabi, Stockholm, Sweden) was dissolved in distilled water to a final concentration of 1 Unit/ml.

Synthetic chromogenic substrate, S-2238 (substrate for thrombin) (Kabi Diagnostika, Stockholm, Sweden). A 25 mM solution was used. Buffer pH 8.4, contained Tris (50 mM), Na_2EDTA $2\text{H}_2\text{O}$ (7.5 mM) and NaCl (175 mM). The formation of p-nitroaniline (pNA) from the substrate was measured spectrophotometrically at 405 nm after the incubation period of 120 seconds and the thrombin concentration calculated from a standard curve plotted for known concentrations of thrombin.

Human umbilical cords were sampled at the time of delivery and the veins were immediately rinsed with physiological saline in order to remove all blood. They were kept on ice until they were tanned with glutardialdehyde or until the experiments were performed. All thrombin determinations were done on the day of the sampling of the cord.

Tanning of the umbilical cord veins with glutardialdehyde was performed essentially according to Dardik & Dardik (11). The veins were mounted on glass rods with the diameter of 5 mm and then treated for 48 hours in the glutardialdehyde solution. Dissection of the cord veins was avoided to minimize mechanical trauma to the endothelial lining. The tanned vein segments were thoroughly rinsed with distilled water, remounted on the glass rods and then stored in 50% ethyl alcohol.

Heparin incubation of the glutardialdehyde treated veins was done as follows: the vein segments were filled with a heparin solution (5000 IE/ml), occluded in both ends and incubated for 24 hours. The heparin solution was then decanted and the veins were again thoroughly rinsed with physiological saline after which they were again mounted on the glass rods for storage in ethyl alcohol. Before testing, as described later, the veins were thoroughly rinsed with saline and then incubated with human plasma for one hour.

EXPERIMENTAL PROCEDURE

All incubations and experiments were performed at room temperature. Twenty cm long segments of human umbilical cords were used in the experiments. They were incubated with 1 ml of the thrombin/albumin solution for 15 minutes, during which time they were rotated on a slanted turntable at 5 rpm. The thrombin/albumin solution was then poured off and the loss of thrombin was calculated by measuring the concentration of thrombin in the solution before and after exposure to the vein wall. The segments of the veins were then rinsed with 2 x 20 ml of saline at room temperature. The surfaces were thereafter exposed to a solution of substrate S-2238 and buffer (0.3 and 0.7 ml respectively) for 120 seconds. The presence of surface bound thrombin was established by determining the concentration of pNA in the solution after its removal from the vein.

After rinsing with saline as above the endothelium was exposed to the antithrombin III solution for 15 minutes and the surface bound thrombin activity was again determined. The surface bound thrombin was expressed in arbitrary units per cm^2 of the vein wall.

The different groups of vessels investigated are as follows:

Group I: Fresh umbilical cord veins

Group II: Glutardialdehyde treated umbilical cord veins

Group III: Heparin treated glutardialdehyde treated umbilical cord veins. Stored in alcohol after the heparin incubation.

Group IV: Heparin treated glutardialdehyde treated umbilical cord veins. Not stored in ethyl alcohol after the heparin incubation.

Statistics. The results are given as mean values $\pm$ S.D. All values were equally distributed. Student's t-test was used for the calculations and the significant level was set at $p < 0.02$.

RESULTS

A loss of approximately 50% of the thrombin activity in the thrombin/albumin solution was always observed when the different umbilical vein surfaces were exposed to this solution. This loss proved to be quite reproducible within each group, after correction for variations in total surface areas. No loss of activity occurred during storage of the thrombin/albumin solution.

TABLE I

Thrombin disappearance from the solution after 15 min. exposure to the surface. (For description of the different groups see under Experimental procedure.

	Group I n = 10	Group II n = 11	Group III n = 7	Group IV n = 4
Thrombin loss Units/cm ²	0.35 [±] 0.10	0.47 [±] 0.12	0.38 [±] 0.10	0.19 [±] 0.04

There were no significant differences among the first three groups, i.e. the fresh veins, those treated with glutardialdehyde and stored in ethyl alcohol and those treated with heparin in addition to the glutardialdehyde treatment and stored in ethyl alcohol. In the fourth group, consisting of veins treated with heparin after glutardialdehyde treatment and alcohol storage, but then rinsed with saline only, the loss of thrombin activity in the solution was significantly lower.

In all the groups, enzymatically active thrombin was detected on the vein surfaces after their exposure to the thrombin/albumin solution. Group IV showed consistently lower surface bound thrombin activity than the other three groups which were indistinguishable.

TABLE II

Thrombin adsorption and inactivation by the different surfaces.

	Group I n = 10	Group II n = 11	Group III n = 7	Group IV n = 4
Thrombin adsorption ₂ Units/cm ²	0.36 [±] 0.07	0.28 [±] 0.11	0.28 [±] 0.15	0.18 [±] 0.11
Remaining thrombin after incubation with AT III	0.07 [±] 0.02	0.24 [±] 0.08	0.07 [±] 0.03	0.12 [±] 0.12

After exposure of the veins with surface adsorbed thrombin to the antithrombin III solution, the thrombin activity proved to be almost completely inhibited on the fresh veins and also on the veins treated with heparin and stored in ethyl alcohol. The veins treated with glutardialdehyde and those additionally treated with heparin but not stored in ethyl alcohol, were found to retain nearly all their surface bound thrombin activity after exposure to the antithrombin III solution (Table II).

DISCUSSION

The present investigation demonstrates that the endothelium of the fresh human umbilical cord vein has a pronounced capacity to adsorb thrombin and subsequently inactivate the immobilized enzyme on contact with antithrombin III. The results on adsorption of enzymatically active thrombin on the endothelium are in agreement with those of Awbrey (5), who reported specific receptors on the endothelial cells of umbilical cord vein for binding thrombin without involving the active site of the enzyme.

Larsson et al recently showed that a heparinized surface stabilized with glutardialdehyde exerted similar thrombin adsorption and neutralization properties as the endothelium (9). A number of artificial surfaces prepared from various glucosaminoglycans were also shown to bind thrombin. Sulphate groups originating from glucosaminoglycans normally present in the endothelium (1) may well create the binding capacity for the cationic thrombin molecule. The data on the surface bound activities are relative as conditions for measuring the surface bound activities did not allow for strict quantitation in terms of NIH units. It is noteworthy, however, that comparable figures are obtained for the amount of thrombin adsorbed on the endothelium and on the artificial glucosaminoglycan surfaces.

The surface bound thrombin could not be inactivated on the vein segments that had been treated with glutardialdehyde and stored in ethyl alcohol. This function, however, could be restored by incubation with heparin followed by storage in ethyl alcohol, but not by heparin incubation alone. The endothelial cell surface contains specific binding sites for heparin (4) and incubation with heparin could thus result in a heparin coating of the endothelium. The beneficial effect of the storage in ethyl alcohol may be explained in terms of stabilization, i.e. the structures responsible for the heparin binding might precipitate in the presence of alcohol leading to a more stable binding. Accordingly, the endothelial surface treated in such a way might be described as a type of heparinized surface.

The molecular mechanisms involved during inactivation of surface adsorbed thrombin on the fresh umbilical cord veins remain unclear. It is well known that heparin owes its anticoagulant effect to catalytic acceleration of the inhibitory

action of antithrombin III. On basis of experiments with different modified surfaces prepared from heparin fractions and heparin analogues, Larsson et al concluded that heparin containing fractions with significant anticoagulant activities are crucial for achieving an artificial surface with the capacity of inactivating surface adsorbed thrombin. Hence, a surface prepared from e.g. heparan sulphate proved to be highly thrombogenic due to its ability to adsorb thrombin in combination with its inability to inactivate the adsorbed enzyme (9). Although the endothelium has been shown to be a compartment for exogenous heparin (4), there is no evidence that endogenous heparin should be present on the endothelium. In view of the present results and the fact that heparan sulphate is found on the surface of endothelial cells (1) it is tempting to speculate that heparan sulphate might have a potent heparin-like effect in its native environment although the purified polysaccharide exerts a very low anticoagulant effect (12, 13).

The present investigation establishes the ability of the umbilical cord vein endothelium together with antithrombin III, to inhibit adsorbed thrombin. Preliminary experiments indicate that the human saphenous vein possesses the same properties. Heparin coated polyethylene has been demonstrated to be regenerative in this function (9) suggesting that the endothelium may act as an immobilized anticoagulant.

The thrombin inhibiting function of the endothelium was destroyed by tanning with glutardialdehyde, a treatment which is applied to umbilical cord veins being used as vascular substitute in clinical surgery (14). Such a prosthesis may thus have inherent thrombogenic properties due to its incapacity to neutralize thrombin adsorbed from the circulation. The manufacturers of the umbilical cord vein grafts propose incubation with heparin immediately prior to their implantation in order to improve the non-thrombogenic properties. The present finding indicates that such a treatment is of minor value unless the heparin in some way is stabilized on the surface.

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IN VITRO EVALUATION OF A BIOLOGIC GRAFT
SURFACE. EFFECT OF TREATMENT WITH CONVENTIONAL AND LOW
MOLECULAR WEIGHT (LMW) HEPARIN.

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ABSTRACT

Human umbilical vein grafts were treated with either conventional or LMW heparin, followed by exposure to alcohol. The grafts were investigated for their ability to adsorb and inactivate thrombin, and comparison was made with non-heparinized and saline-alcohol treated grafts and grafts supplied with a covalently bonded layer of conventional heparin. In addition, the effect of protamine exposure to heparin-alcohol and LMW heparin-alcohol treated grafts as well as native human umbilical veins (HUV) was studied.

Native HUV and heparin treated graft surfaces adsorbed and inactivated thrombin, whereas non-heparinized and saline-alcohol treated grafts inactivated surface-bound thrombin to only a small degree. Surface-bound LMW heparin exhibited a significantly lower ability to inactivate thrombin as compared with conventional heparin, but LMW heparin-alcohol surfaces were better than non-heparinized ones.

Protamine treatment of "heparinized" surfaces impaired the thrombin inhibiting ability of the heparin-alcohol surface, whereas this property was totally abolished for the LMW heparin-alcohol surface.

The findings indicate that LMW heparin, despite its weaker thrombin inhibiting capacity, may be an alternative to conventional heparin, for "heparinizing" the human umbilical vein graft. Protamine exposure may be potentially harmful for a heparin treated surface, although protamine concentrations used in the present in

Key words: Thrombin uptake and inhibition, heparin, LMW heparin, human umbilical vein graft, protamine, heparin treated surfaces.

vitro study may not be reached in vivo. The native HUV was not at all affected by protamine exposure regarding its ability to inactivate thrombin.

INTRODUCTION

The intact vascular endothelium has a number of properties contributing to a blood compatibility superior to that of any vascular graft material currently available. As has been demonstrated on the human umbilical vein (HUV) (1) and on the canine and porcine aorta (2), one of these properties is the ability to adsorb and inactivate thrombin. This heparin-like activity of the endothelium, which may be attributed to surface-located glucosaminoglycans (3, 4), is destroyed when HUVs are treated with glutaraldehyde, but is restored after treatment with heparin and alcohol (1). HUV grafts (Dardik Biograft, Meadox) "heparinized" in this way have a significantly reduced thrombogenicity in vivo (5). Stabilized covalent bonding of heparin according to a new method (6) to PTFE results in a decreased thrombogenicity also of this graft material (7).

Theoretically and experimentally low molecular weight (LMW) heparin is associated with a lesser influence on initial hemostasis than conventional heparin (8), and is only partly counteracted by protamine (9). These properties could be an advantage in vascular surgery. The aim of this study was therefore to compare the thrombin-inhibiting capacity in vitro of Biografts treated with conventional or LMW heparin, and to evaluate the effect of protamine on thus "heparinized" surfaces. In addition, heparin was bonded covalently to Biograft surfaces, which were subsequently studied with respect to their thrombin-inhibiting capacity.

MATERIAL AND METHODS

Human albumin, 20% (KABIVITRUM, Stockholm, Sweden). Diluted to 4% in physiologic saline.

Thrombin, Topostasin Roche (Hoffman-La Roche, Basle, Switzerland). One ampoule (3000 NIH U) was dissolved in 30 ml of distilled water and stored at -70°C . The activity given by the manufacturer was accepted. Prior to the experiments the stock thrombin solution was diluted in human albumin to a concentration of 20 NIH U/ml.

Conventional heparin, (KABIVITRUM, Stockholm, Sweden). Mean MW 15000 (4000-25000), porcine mucosa. Water solution. 170 IU/mg antifactor Xa and 170 IU/mg APTT activity.

Low molecular weight heparin fragment, LMW heparin (KABIVITRUM, Stockholm, Sweden). Batch No DxN11; MW 4000-5000, porcine mucosa. 165 IU/mg antifactor Xa and 40 IU/mg APTT activity.

Protamine chloride, 10 mg/ml (KABIVITRUM, Stockholm, Sweden). Water solution.

Human antithrombin III, (KABIVITRUM, Stockholm, Sweden). Water solution, final concentration 1 Unit/ml.

Synthetic chromogenic substrate, S-2238 (substrate for thrombin) (KABI, Diagnostika, Stockholm, Sweden). The specificity of S-2238 for thrombin is high. The rate of hydrolysis caused by other plasma proteolytic enzymes is less than 2% of that caused

by thrombin when equimolar amounts of the enzymes are compared (10). A 25 mM solution was used. The buffer solution of pH 8.4 contained Tris (50 mM), $\text{Na}_2\text{EDTA} \times 2 \text{H}_2\text{O}$ (7.5 mM) and NaCl (175 mM). The formation of p-nitroanilin (pNA) from the substrate was measured spectrophotometrically at 405 nm after the incubation period of 120 seconds and the thrombin concentration calculated from a standard curve plotted for known thrombin concentrations. Human umbilical veins (HUV). Umbilical cords were sampled at the time of delivery. Veins were immediately rinsed with physiologic saline and frozen at -20°C until the day of the experiment.

Human umbilical vein grafts, Dardik Biograft (Meadox) were provided by Meadox Medicals through Astra Meditec, Gothenburg, Sweden.

Heparinization procedures:

Heparin and alcohol treatment of Biograft (conventional or LMW heparin) was performed as described earlier (1). Briefly, grafts were incubated in the heparin solution (5000 IU/ml antifactor Xa activity for both conventional and LMW heparin) for 12 hours, and then stored in ethyl alcohol 50%.

Covalent heparin bonding to Biograft, was performed by IRD Biomaterial AB, Stockholm, Sweden and has been described in detail elsewhere (6).

Experimental procedure:

All experiments were performed at room temperature using a method which has been described in detail earlier (1). Segments of the grafts being tested measured about 10 cm in length and the surface area averaged $15\text{--}20 \text{ cm}^2$. Solutions were pipetted into the segments in 1 ml aliquots, whereupon the vessels were occluded at both ends and rotated on a slanting turntable. All experiments were started by exposing the grafts to citrated human normal plasma for one hour. After 15' exposure to the thrombin/albumin solution, surface-bound thrombin was determined by exposing to the chromogenic substrate/buffer solution for 2 minutes before and after the incubation with antithrombin III. The incubation period for antithrombin III was 15 minutes. In addition, the loss of thrombin from the solution was calculated by measuring the concentration before and after exposure to the surface. All values for surface-confined thrombin are given as estimated NIH U/ cm^2 of the surface. In those cases where the effect of protamine was studied, the grafts were exposed to the protamine solution (10 mg/ml) for 15' before the incubation with thrombin.

Surfaces studied:

1. Untreated Biograft.
2. Saline-alcohol treated Biograft.
3. Heparin-alcohol treated Biograft, before and after protamine exposure.
4. Covalently-heparinized Biograft.

5. LMW heparin-alcohol treated Biografts, before and after protamine exposure.
6. Fresh HUV, before and after protamine exposure.

Statistical methods

The results are given as means $\pm$ S.D. Student's t-test was used for the statistical calculations and differences were considered significant when $p < 0.05$.

RESULTS

The disappearance of thrombin from the solution and the subsequent adsorption of enzymatically active thrombin on the surfaces is shown in Table I. The untreated and saline-alcohol treated Biografts caused a significantly greater loss from the solution than all other surfaces ($p < 0.001$ when compared to heparin-alcohol). There was no difference between those two Biograft surfaces regarding either loss from solution or uptake on the surface. Comparing conventional and LMW heparin, there was a greater loss from the solution after contact with the LMW heparin surface ($p < 0.025$).

TABLE I

Disappearance of thrombin from solution and measurable thrombin activities on the various Biograft surfaces and the native HUV after exposure.

Surface	Loss of thrombin NIH U/cm ²	Uptake of thrombin NIH U/cm ² (est.)
Untreated Biograft	0.89 $\pm$ 0.21 n=8	0.92 $\pm$ 0.28 n=8
Saline-alcohol Biograft	1.00 $\pm$ 0.04 n=5	0.76 $\pm$ 0.06 n=5
Heparin-alcohol Biograft	0.35 $\pm$ 0.09 n=5	0.40 $\pm$ 0.17 n=6
Covalently-heparinized Biograft	0.45 $\pm$ 0.09 n=2	0.65 $\pm$ 0.01 n=2
LMW heparin-alcohol Biograft	0.49 $\pm$ 0.07 n=5	0.72 $\pm$ 0.18 n=5
Native HUV	0.35 $\pm$ 0.10 n=10	0.36 $\pm$ 0.07 n=10

There was a greater uptake on LMW heparin-alcohol treated Biograft surfaces than on surfaces treated with conventional heparin ($p < 0.01$). Native HUV behaved as heparin-alcohol treated Biografts.

Table II shows the antithrombin capacitating ability of the various surfaces. The two modifications of non-heparinized Biograft surfaces did not differ, almost 80 % of the initially found thrombin still being present after exposure to AT III. All other surfaces developed a significantly better ability to inactivate thrombin. On Biografts treated with conventional heparin only about 10 % of the initially bound thrombin was encountered after incubation with AT III. Biografts treated with LMW heparin also inactivated thrombin but to a lesser extent than when conventional heparin was used ($p < 0.005$). The fresh HUV exhibited a significantly lower thrombin inactivating effect than the heparin-alcohol treated surface ($p < 0.025$). Finally, Biografts with covalently bonded heparin seemed to display much the same properties as grafts with the other variants of heparin treatment.

TABLE II

Thrombin inhibition by the various Biograft surfaces after exposure to AT III, compared to native HUV.

Surface	Uptake of thrombin NIH U/cm ² (est.)	Residual thrombin % of initial value	n
Untreated Biograft	0.92 $\pm$ 0.28	79 $\pm$ 17	8
Saline-alcohol Biograft	0.76 $\pm$ 0.06	77 $\pm$ 19	5
Heparin-alcohol Biograft	0.40 $\pm$ 0.17	7 $\pm$ 10	6
Covalently- heparinized Biograft	0.65 $\pm$ 0.01	32 $\pm$ 9	2
LMW heparin- alcohol Biograft	0.72 $\pm$ 0.18	30 $\pm$ 12	5
Native HUV	0.36 $\pm$ 0.07	21 $\pm$ 5	10

The effects of treating heparinized Biografts and fresh HUV with protamine are outlined in Table III. Heparin-alcohol treated Biografts showed a greater loss from the solution and an increased uptake on the surface after protamine exposure ($p < 0.025$ and 0.05 respectively). The thrombin-inactivating capacity was also impaired ($p < 0.005$) but was still better than for untreated Biograft surfaces ($p < 0.005$). Biografts treated with LMW heparin-alcohol showed a behaviour opposite to that of grafts treated with conventional heparin after protamine incubation, i.e. a reduction of the loss from the solution ($p < 0.05$) and of the adsorption on the surface ($p < 0.005$). As for conventional heparin, the thrombin-inhibiting capacity was impaired ($p < 0.005$), and statistically was not better than untreated Biografts, after protamine treatment. Protamine did not affect either adsorption on or the inactivating capacity of the fresh human umbilical

vein.

TABLE III

Effect of protamine on thrombin disappearance from solution and thrombin adsorption and inhibition by the various Biograft surfaces and the native HUV.

Surface	Loss from solution NIH U/cm ²	Uptake NIH U/cm ² (est.)	Residual % of initial	n
No prot.	0.35±0.09	0.40±0.17	7±10	6
Heparin-alcohol	**	*	***	
Protamine	0.68±0.30	0.63±0.23	46±10	5
No prot.	0.49±0.07	0.72±0.18	30±12	5
LMW heparin-alcohol	*	***	***	
Protamine	0.40±0.07	0.28±0.05	66±10	5
No prot.	0.35±0.10	0.36±0.07	21±5	10
Native HUV	n.s.	n.s.	n.s.	
Protamine	0.41±0.04	0.31±0.07	26±2	5

*=p<0.05, **=p<0.025, ***=p<0.005

DISCUSSION

The vascular endothelium has specific binding sites for thrombin, where it is immobilized but still enzymatically active (11, 12). The exact nature of these binding sites is unknown, one possibility being different glucosaminoglycans (13, 14). This assumption is supported by the fact that an artificial surface with different glucosaminoglycans adsorbs thrombin to about the same extent as the vascular endothelium (15). The immobilized thrombin is inactivated on exposure to antithrombin III (1), defibrinogenated plasma or, at a slower rate, to a modified Ringer's solution (2). Besides antithrombin III, an additional plasma factor was considered necessary for the rapid phase of inactivation (16). In order to produce vascular substitutes, human umbilical veins are tanned with glutaraldehyde (17). This procedure does not impair their ability to adsorb thrombin, but their thrombin inhibiting capacity is destroyed, as we showed in a previous study (1). This observation is confirmed by the present investigation which also demonstrates that excessive rinsing of the grafts with saline and alcohol and hence a more total remo-

val of glutaraldehyde residues does not restore the thrombin inhibiting capacity. The results in this and a previous study (1) on heparin and alcohol treated Biografts permits the conclusion that in this model the heparin molecule interacts with antithrombin III. In addition, although only two grafts were available in this group for testing, and therefore statistical calculations are not possible, covalent bonding of heparin to Biografts seems to add the same property to the surface.

Conventional heparin is a large and heterogenous substance concerning molecular weight, chemical composition and effects on the haemostatic system. The larger fractions are probably responsible for the risk of bleeding complications and the effect on platelet aggregation (18, 19). Low molecular weight fragments of heparin can be obtained by nitrous acid degradation of standard heparin (20). A high affinity for antithrombin, which is necessary for the effect as an anticoagulant (21), is also important for the effects on platelets, fragments with high affinity for antithrombin III having the least platelet activating effect (18, 20). Salzman therefore suggested that, when heparinizing artificial surfaces, in order to achieve improved thromboresistance, a low molecular weight heparin with high affinity for antithrombin III should be used (22).

The thrombin inhibiting activity of heparin fragments, like the platelet activating effect, is dependent on molecular weight. Consequently, the smaller the molecule, the weaker its antithrombin capacitating ability (21). The fragment used in the present study has a high affinity for antithrombin III and an antifactor Xa/APIT ratio of 4.1:1, compared to a ratio of 1:1 for conventional heparin. The results obtained in this investigation with LMW heparin located on the surface of Biografts indicate an antithrombin cofactor effect, although it is weaker than with conventional heparin, and hence the effects referred to above are significant also in this experimental situation. Whether this effect is enough to increase thromboresistance in the in vivo situation remains to be determined.

Protamine chloride, a neutralizing agent of heparin, acts by binding to the heparin molecule and dissociating the heparin-antithrombin III complex (23). In vitro, this inactivation is dependent on the molecular weight of the heparin preparation, requiring a length of the polysaccharide chain which exceeds the site of its AT III binding sequence (9). Thus, considerably higher protamine concentrations are needed to abolish the APIT activity of LMW heparin and the FXa activity can not be extinguished. Protamine treatment of vascular endothelium or surfaces supplied with different glucosaminoglycans in vitro, has been demonstrated to cause an inhibition of the uptake of thrombin (16).

In our study this finding was only related to Biografts treated with LMW heparin. In contrast, the uptake was increased on surfaces treated with conventional heparin. This may be explained in terms of stability of the binding and concentration of the anticoagulant on the surface. Thus, on Biografts, the heparin-alcohol treatment leads to a high concentration of heparin molecules on the surface, at least a portion of these being more loosely bound (24). Protamine may interact with these heparin molecules, and the heparin-protamine complex be rinsed away,

leaving binding sites on the surface for thrombin to react with. The surface then acts more like a non-heparinized one, but since the heparin concentration initially was high, active heparin molecules are still present on the surface after exposure to protamine. Thus, the net result is that of an impaired, but still existing antithrombin cofactor activity.

The difference in behaviour after protamine incubation between the Biograft surfaces treated with conventional or LMW heparin, may be explained as a function of the weaker thrombin inhibiting capacity of the fragment, the effect of this being further amplified by the fact that both heparins were dosed according to anti-Xa-activity. Another possible explanation may be that the fraction of loosely bound heparin may be smaller for LMW than for conventional heparin. Thus, the LMW heparin-protamine complex is still present on the surface, resulting in a decreased uptake of thrombin and a destroyed capacity to inactivate thrombin. This is a finding opposed to earlier results, showing LMW heparin to be counteracted only by higher protamine doses, than those active against conventional heparin (9).

The clinical relevance of the effects of protamine exposure to heparin treated Biograft surfaces is doubtful, since clinically administered protamine doses are adjusted to inactivate previously injected circulating heparin. Thus, protamine concentrations will not reach those used in the present in vitro experiments.

Fresh umbilical veins are not at all affected as regards their thrombin inactivating capacity subsequent to protamine treatment. This is contrary to the effect of protamine on porcine aorta (16). A possible explanation may be that there is a difference in the efficacies of the antithrombotic activity of the human umbilical vein and the porcine aorta. The defence mechanisms of the umbilical vein may be stronger and even regenerative after protamine incubation, or the thrombin inhibiting capacity may be caused by different glucosaminoglycans. This idea is supported by the fact that thrombosis of the HUV is extremely uncommon (25). The finding for the HUV is interesting, and prompts further investigation.

The present investigation allows the following conclusions:

The present in vitro method, imaging one facet of the thromboresistance of the vascular endothelium, is a valuable tool in assessment of the thrombogenicity of vascular graft materials prior to more expensive and time-consuming in vivo implantations.

LMW heparin, lacking some of the unwanted effects of conventional heparin, may, despite its weaker thrombin inhibiting activity, be a realistic alternative when preparing vascular graft surfaces with an anticoagulant.

Protamine, sometimes administered during vascular surgery to reverse the effect of systemic heparin, is potentially harmful for heparin treated vascular grafts.

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THROMBIN UPTAKE AND INHIBITION ON HEPARINIZED
POLYTETRAFLUOROETHYLENE (PTFE) GRAFTS AND
NATIVE SHEEP VESSELS

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Running title: Thrombin inhibition by PTFE grafts.

ABSTRACT

Non-heparinized and covalently-heparinized PTFE grafts, sheep aorta, carotid artery and jugular vein were evaluated according to their ability to adsorb and inactivate thrombin. Thrombin concentrations on surfaces and in solutions were assayed by use of a chromogenic peptide substrate specific for thrombin.

When the various surfaces were exposed to the thrombin solution considerable losses of enzymatically active thrombin were encountered from the solutions, except in the case with non-heparinized PTFE. Covalently-heparinized PTFE and jugular veins adsorbed thrombin, which was inactivated on subsequent contact with antithrombin III. Despite the same loss from the solution as was encountered with veins arteries exhibited significantly smaller amounts of surface-bound thrombin. Protamine treatment of covalently-heparinized PTFE almost totally abolished the ability to adsorb thrombin.

It is concluded that 1) arteries possess a stronger thrombin inhibitory capacity than veins, possible explanations to this being a different glucosaminoglycan or the presence of antithrombin III in the arterial wall or a combination of both and 2) that heparinizing PTFE results in a pronounced ability to inactivate thrombin, which may increase the thrombo-resistance of this graft material.

INTRODUCTION

Expanded polytetrafluoroethylene (PTFE) was introduced as a material for vascular prostheses about ten years ago (21, 7). Since then, it has been increasingly used for femoropopliteal bypass grafting when the autologous saphenous vein is not available.

Recent studies indicate that, although PTFE grafts function satisfactorily in low-risk situations, i.e. with the distal anastomosis above knee and/or good run-off, the performance is not as good as for the autologous vein in situations of high peripheral resistance and low flow-rates (2, 10, 17). Two recent, prospective studies showed PTFE to be inferior to saphenous vein (3) and the modified human umbilical

vein (Dardik Biograft) (13) in femoro-popliteal reconstructions with the distal anastomosis below the knee.

Heparinized Biografts and covalently-heparinized PTFE perform better than non-heparinized(5, 14). As regards Biografts one explanation to this is an in vitro ability to adsorb and inactivate thrombin (4). Whether this property can be obtained by PTFE grafts is not known.

The aim of the present study therefore was to evaluate the thrombin-inhibiting ability of non-heparinized and covalently-heparinized PTFE and to compare results to those obtained for native sheep arteries and veins.

MATERIAL AND METHODS

Human albumin, 20% (KABIVITRUM, Stockholm, Sweden). Diluted to 4% in physiologic saline.

Thrombin, Topostasin Roche (Hoffman-La Roche, Basle, Switzerland). One ampoule (3000 NIH U) was dissolved in 30 ml of distilled water and stored at -70°C . The activity given by the manufacturer was accepted. Prior to the experiments, the stock thrombin solution was diluted in human albumin to a concentration of 20 NIH U/ml.

Protamine chloride, 10 mg/ml (KABIVITRUM, Stockholm, Sweden). Water solution.

Human antithrombin III, (KABIVITRUM, Stockholm, Sweden). Water solution, final concentration 1 Unit/ml.

Synthetic chromogenic substrate. The chromogenic substrate S-2238 (Kabi Diagnostica, Stockholm, Sweden) used in this study has a high specificity for thrombin, the rate of hydrolysis caused by other plasma proteolytic enzymes being less than 2 % of the rate caused by thrombin comparing equimolar amounts of the enzyme (9). A 25 mM solution was used. The buffer solution with pH 8.4 contained Tris (50 mM), $\text{Na}_2\text{EDTA} \times 2 \text{H}_2\text{O}$ (7.5 mM) and NaCl (175 mM). The formation of p-nitroanilide (pNA) from the substrate was measured spectrophotometrically at 405 nm after the 120 second incubation period and the thrombin concentration calculated from a standard curve plotted for known thrombin concentrations.

Sheep jugular vein and carotid artery were obtained from sheep

during general anesthesia. The vessels were carefully dissected, tributaries ligated, removed and gently rinsed with saline and then stored at -20°C until the day of the experiment.

Sheep aortae were obtained from sheep being used for graft implantation experiments. The animals were exsanguinated and thoracic aortae were dissected out, tributaries ligated and vessels stored at -20°C until the day of the experiment.

PTFE grafts, (Gore-Tex) were provided by Svenska Gore AB, Gothenburg, Sweden. Heparinized grafts with internal diameters of 4 mm and non-heparinized grafts with internal diameters of 6 mm were used. Lengths were adjusted so that the inner surfaces for both heparinized and non-heparinized grafts were equal.

Heparinization procedure:

Covalent heparin bonding to PTFE was performed by IRD Biomaterial AB, Stockholm, Sweden and has been described in detail elsewhere (18). By this method heparin is bonded in a vertical position, leaving a larger amount of active sites free to interact with antithrombin III, than are available after conventional covalent bonding methods.

Experimental procedure:

All experiments were performed at room temperature using a method which has been described in detail earlier (4). Surface areas of the segments of the grafts or vessels being tested averaged $15\text{--}20\text{ cm}^2$. Solutions were pipetted into the segments in 1 ml aliquots, after which they were occluded at both ends and rotated on a slanting turntable. All experiments were started by exposing the grafts/vessels to citrated human normal plasma for one hour. After 15 minutes exposure to the thrombin/albumin solution, surface-bound thrombin was determined by exposing to a chromogenic substrate/buffer solution for 2 minutes before and after incubation with antithrombin III. The incubation period for antithrombin III was 15 minutes. In addition, the loss of thrombin from the solution was calculated by measuring the concentration before and after exposure to the surface. All values for surface-confined thrombin are given as estimated NIH U/ cm^2 of the surface.

In those cases where the effect of protamine was studied, grafts were exposed to the protamine solution (10 mg/ml) for 15 minutes before the incubation with thrombin.

Surfaces studied:

1. Untreated PTFE (non-heparinized).
2. Covalently-heparinized PTFE, before and after protamine exposure.
3. Sheep jugular vein.
4. Sheep carotid artery.
5. Sheep aorta.

Statistical methods

The results are given as means $\pm$ S.D. Student's t-test was used for the statistical calculations and differences were considered significant when $p < 0.05$.

RESULTS

The disappearance of thrombin from the solutions and the subsequent adsorption of enzymatically active thrombin on the surfaces is shown in Table I. Untreated PTFE caused very little losses of thrombin from the solutions and no significant uptake was encountered on the surfaces. Heparinization of PTFE surfaces resulted in considerable losses from the solutions, equal to those encountered with the native sheep vessels. Adsorption on the PTFE surfaces was significantly greater than on native sheep vessels ($p < 0.01$ as compared with sheep jugular vein). Sheep aortae and carotid arteries, despite the same loss as compared with the jugular vein, displayed significantly smaller amounts of amidolytically active thrombin ($p < 0.005$).

TABLE I

Disappearance of thrombin from solution and measurable thrombin activity on the various surfaces after exposure.

Surface	Loss of thrombin NIH U/cm ²	Uptake of thrombin NIH U/cm ² (est.)	n
Untreated PTFE	0.14±0.05	0.02±0.04	5
Cov-hep PTFE	0.41±0.13	1.13±0.17	5
Sheep jugular vein	0.52±0.09	0.72±0.11	4
Sheep carotid artery	0.71±0.01	0.15±0.00	2
Sheep aorta	0.51±0.07	0.07±0.09	6

Table II shows the antithrombin capacitating activity of the various surfaces. Covalently-heparinized PTFE inhibited thrombin to about the same extent as the sheep jugular veins and sheep carotid arteries while sheep aortae readily inactivated the small amounts of thrombin bound to their surfaces.

TABLE II

Thrombin inhibition of PTFE surfaces after exposure to AT III compared to sheep jugular vein, carotid artery and aorta.

Surface	Uptake of thrombin NIH U/cm ² (est.)	Residual thrombin % of initial value	n
Untreated PTFE	0.02±0.04	—	5
Cov-hep PTFE	1.13±0.17	23±10	5
Sheep jug. vein	0.72±0.11	13±7	4
Sheep carotid artery	0.15±0.00	14±7	2
Sheep aorta	0.07±0.09	0	6

The effect of treating heparinized PTFE with protamine is outlined in Table III. This shows that there was a significantly decreased loss from the solution and uptake on the surface ($p<0.005$). The small amount of thrombin that was adsorbed to the surface disappeared after AT III exposure.

TABLE III

Effect of protamine on thrombin disappearance from solution and thrombin adsorption and inhibition by covalently-heparinized PTFE.

Surface	Loss from solution NIH U/cm ²	Uptake NIH U/cm ² (est.)	Residual % of initial	n
No prot.	0.41 [±] 0.13 ***	1.13 [±] 0.17 ***	23 [±] 10	5
Protamine	0.06 [±] 0.03	0.18 [±] 0.10	0	5

*** $p<0.005$

DISCUSSION

Polyethylene catheters with a glutaraldehyde-stabilized heparin layer adsorb and inactivate thrombin (19). Studying various glucosaminoglycan-coated surfaces, Larsson et al. found that only the surface coated with heparin exerted this effect (19). In this respect, the heparinized surface behaved as the vascular endothelium, as has been demonstrated both for the human umbilical vein and canine and porcine aorta (4, 11). It has been shown that normal vessels possess specific binding sites for thrombin, where it is immobilized in an enzymatically active state (1, 20), and it has been speculated that surface-located glucosaminoglycans constitute these binding sites (15, 16). This idea is supported by the results with heparinized tubings and also by the fact that heparin and alcohol treated Biografts exhibit a capacity, equal to that of the human umbilical vein, to inactivate thrombin (4, 11). The covalent bonding method used in the present study, is a modification of the ionic bonding method, resulting in a possibly more stable heparin layer (18), and has also been applied to Biografts. The in vitro results indicated an effect approaching that of heparin-alco-

hol treated grafts (6), in vivo findings in an acute sheep model, however, demonstrating this heparinization method to be unsuitable for the fragile Biograft (5). Since PTFE grafts heparinized in this way showed a decreased thrombogenicity in the same sheep model (14) it seemed logical to assess the in vitro effects of the covalent heparinization method applied to PTFE surfaces. The results of the present study are thus consistent with in vivo findings and demonstrates that covalently-heparinized PTFE exhibits a pronounced thrombin inhibitory capacity. The non-heparinized PTFE showed an insignificant ability to adsorb thrombin, indicating that this artificial surface lacks surface-groups capable of binding thrombin.

As expected, the native vessels showed excellent thrombin inhibiting properties, but there was also a marked difference between veins and arteries. Although the disappearance of thrombin from the solution was the same, a significantly smaller amount of active thrombin was recovered on the surfaces of aortae and carotid arteries than on jugular veins. Even though only two carotid arteries were tested, the results are consistent with those of aortae, and thus it seems reasonable to conclude that differences in harvesting the vessels are not responsible for the observed differences between veins and arteries. Previous investigations have demonstrated that antithrombin III is necessary for the inactivation of surface-confined thrombin to occur (12). The results of the present study indicate an inhibitory function of arterial endothelium acting even in the absence of AT III and it may be suggested that this is due to a glucosaminoglycan independent of AT III, or, to AT III present in the vessel wall. Human umbilical vein endothelial cells have been reported to produce AT III (8), however, the functions of this vein are more like those of arteries, transporting oxygenated blood to the fetus. Dryjski et al (12) studied uptake and inhibition of thrombin on canine and porcine aortae and found small but significant amounts of thrombin after exsanguination. After exposure to a thrombin solution, they recovered surface-bound thrombin activities of a concentration resembling these found on veins in the present study. The significance of this difference, as compared with the present study, is difficult to evaluate, one possible explanation being the species difference.

Protamine chloride, a neutralizing agent of heparin, acts by binding to the heparin molecule and dissociating the heparin-antithrombin III complex (22). Protamine treatment of vascular endothelium or surfaces supplied with different glucosaminoglycans in vitro has been demonstrated to inhibit the uptake of thrombin (12). The same results were encountered with covalently-heparinized PTFE in the present study. Thus, there was a significantly smaller loss from the solution and uptake on the surface after protamine treatment. The small amount of thrombin recovered on the protamine incubated surface was totally abolished after AT III exposure, which indicates that a few active heparin molecules were still present on the surface after protamine exposure. It may thus be concluded that not even the high protamine concentration used in the present study was capable of totally inactivating the surface-bound heparin. This is of importance, considering the fact that systemically administered protamine doses are adjusted to inactivate previously injected, circulating heparin. Thus, protamine concentrations in vivo will never reach the level used in the present in vitro study.

The present investigation allows the following conclusions:

Covalently-heparinized PTFE has the ability to adsorb and inactivate thrombin, a property that probably contributes to an increased thrombo-resistance.

Sheep arterial endothelial cells exhibit a thrombin inhibitory function that is different from and probably also more efficient than that of veins.

ACKNOWLEDGEMENTS

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Decreased Acute Thrombogenicity of Human Umbilical Veins after Heparin and Alcohol Treatment¹

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Key Words. Thrombus · Human umbilical vein grafts · Heparin · Carotid arteries · Dog

Abstract. Nine glutaraldehyde-tanned human umbilical veins (GAUV) were incubated in heparin followed by storage in ethyl alcohol 50% (HAUV). These grafts were compared to five GAUV flushed with a heparinized solution without subsequent treatment in alcohol (HsAUV) and four nonheparinized GAUV (NHUV) placed in the contralateral carotid arteries of dogs for approximately 3 h. The thrombogenicity was evaluated by measuring the platelet adhesion and aggregation (⁵¹Cr), fibrinogen accumulation (¹²⁵I-fibrinogen), the thrombus-free surface and the thrombus weight. The HAUUV had the lowest ⁵¹Cr radioactivity. The means of thrombus-free surface and thrombus weight for the grafts were as follows: HAUUV, 82% and 0.3 g (n = 9); HsAUV, 53% and 0.6 g (n = 5); NHUV, 13% and 1.2 g (n = 4). The HAUUV showed a lower thrombogenicity than both the NHUV and the HsAUV grafts.

Glutaraldehyde-tanned human umbilical veins (GAUV) have been used as arterial substitutes with some success [5] but discouraging reports about poor performance of this material have appeared in the literature [6, 8, 12]. The thrombogenicity of the graft material is an important factor in early failures [3]. It has recently been demonstrated [2] that fresh human umbilical veins inactivate thrombin but this function was lost when the

veins were tanned with glutaraldehyde. The GAUVs regained this particular property after treatment with heparin and storage in ethyl alcohol 50%. Interestingly, the GAUVs treated with heparin, without subsequent storage in alcohol did not seem to inactivate thrombin.

The purpose of this study was to determine the acute thrombogenicity of heparin-alcohol umbilical veins (HAUV) and compare them to glutaraldehyde umbilical veins without heparin (NHUV) and glutaraldehyde umbilical veins treated without heparin

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(NHUV) and glutaraldehyde umbilical veins treated with heparin but without alcohol (HsAUV).

Materials and Methods

9 mongrel dogs of either sex with an average weight of 18 kg were studied. Autologous platelets were labelled with chromium-51 (^{51}Cr) and reinfused the day before the experiment [1]. Homologous ^{125}I -fibrinogen (^{125}I -fib) [11] was given intravenously prior to the operation. Hematocrit, platelet count and fibrinogen were determined at the beginning and at the end of the procedure. The animals were anesthetized with sodium pentobarbital (Mebumal®), intubated and kept on a respirator at room air. Umbilical vein bypass grafts measuring 20 cm in length were placed in the carotid arteries in a loop fashion. The anastomoses were performed end-to-side with running 6-0 polypropylene suture (Prolene®, Ethicon Inc.) and the bypassed segment of the artery was ligated adjacent to each anastomosis. A dose of 1 mg/kg i.v. of heparin (Kabi AB, Stockholm, Sweden) was given before clamping and reversed with protamine (Protamin, Kabi AB) after the last anastomosis was performed. APTT determinations before and after heparin as well as after protamine administration were obtained in order to ensure an adequate heparinization and reversal.

Using a proximal, external electromagnetic flow probe and a distal arterial occluder, the grafts were perfused at 50 ml/min in order to accelerate thrombus formation. The bypass loops were isolated with lead shields in order to avoid interference from the radioactivity emitted from the body of the animal. Determinations were made every 5 min for 200 s using an external detector (sodium iodide thallium-activated scintillation counter). The radioactivity was recorded in counts per second after subtracting the background radioactivity. Blood samples were obtained at 15-min intervals throughout the experiment for determination of radioactivity. There was a marked variation in the results of platelet tag between dogs; therefore, a ratio of the counts of the grafts to counts per gram of blood was calculated. After the clamps were removed, there was a lapse of 10–20 min before the first measurement of radioactivity could be done. This time was necessary for hemostasis and installing the

lead shields. Grafts were removed approximately 3 h later and the patency, thrombus-free surface (TFS) and weight of the thrombus material were recorded. The TFS was calculated using a grid on high resolution photographs of the luminal surface [3].

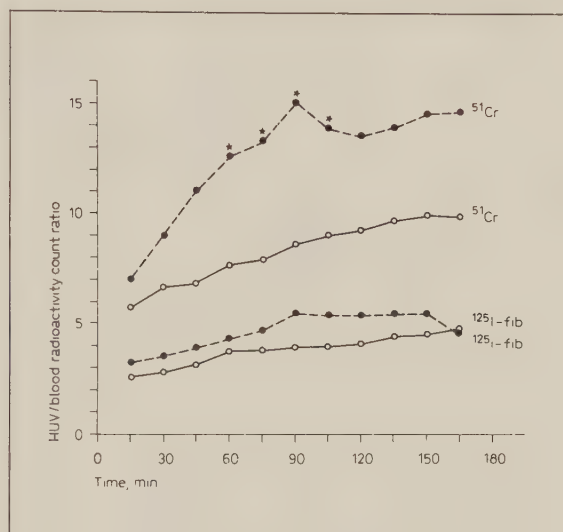
Glutaraldehyde human umbilical veins (Meadox®-Dardik Biograft®) measuring 5 mm in diameter and 20 cm in length were used. The NHUV grafts were flushed with 3 liters of sterile saline solution (Natriumchloride 9 mg/ml, ACO, Sweden) just before the operation. The HsAUVs were flushed with 3 liters of saline solution, each liter containing 10,000 U of heparin (5,000 IE/ml) (Kabi, AB), followed by injection of 30,000 U of heparin (5,000 IE/ml) directly into the lumen and then flushed out with saline. The HAUUVs were flushed with 3 liters of saline, then, filled with a heparin solution (5,000 IE/ml), occluded at both ends and incubated for 12–24 h. The heparin was then decanted; the grafts were again flushed with a saline solution, mounted on the glass rods and stored in ethyl alcohol 50% for a variable period of time from 24 h to 3 days. Before surgery the veins were also irrigated with 3 liters of a sterile saline solution. Using tritium-labelled heparin, a similar radioactivity per luminal surface area was found after incubation with heparin with or without subsequent alcohol treatment as well as after flushing the grafts with a heparinized solution as performed in these experiments [7].

In the first part of this study four HAUUVs were compared to four NHUVs placed in the contralateral side (group I). In the second part five HAUUVs were compared to five HsAUVs also placed in the contralateral side (group II). The data are given as mean $\pm$ SE. The significance of difference was calculated by Student's *t* test.

Results

No significant change was noticed in the hematocrit, platelet count and fibrinogen during the experiments. At the time of the first determination, the vein graft/blood ratio for ^{51}Cr was similar for both grafts in group I (fig. 1), thereafter a sharp increase in the ^{51}Cr uptake, which peaked after 90 min and then stabilized was noticed in the NHUVs. A de-

Fig. 1. Uptake of ^{51}Cr and ^{125}I -fibrinogen by heparin-alcohol (○) and nonheparinized (●) umbilical veins during 3 h of observation (mean of four experiments). * $p < 0.05$.



pression in the curve, probably related to embolization of thrombus material, was observed. There was a slow increase of ^{51}Cr radioactivity in the HAUV grafts. A significant difference was found at 60, 75, 90 and 105 min ($p < 0.05$). The graft/blood count ratio for ^{125}I -fibrinogen showed a progressive increase in both types of grafts and then stabilized between 60 and 90 min. Even though there was a higher ^{125}I -fibrinogen activity in the NHUV grafts, no significant difference could be demonstrated.

Two out of four NHUVs thrombosed 90 and 120 min after protamine administration whereas all HAUVs remained patent. The results regarding TFS and thrombus weight are summarized in table I. There was a significant difference in the TFS ($p < 0.05$ and thrombus weight ($p < 0.05$).

The overall decreased radioactivity in group II was due to the fact that in two of the

Table I. Group I: heparin-alcohol (HAUV) versus nonheparinized human umbilical veins (NHUV)

	TFS, %	Thrombus weight, g	Patency %
HAUV (n = 4)	*75 ± 5	*0.5 ± 0.2	100
NHUV (n = 4)	13 ± 9	1.2 ± 0.4	50

Results after 3 h of observation. Mean and ± SE of four experiments.

* $p < 0.05$.

experiments the blood flow was not restricted, but the data were included since in the same animal the two different types of grafts, i.e. HAUV versus HsAUUV, were compared. The HsAUUV grafts had a higher count ratio for ^{51}Cr and the difference was significant at 135, 150 and 165 min ($p < 0.05$). The ^{125}I -fibrinogen uptake was similar in both

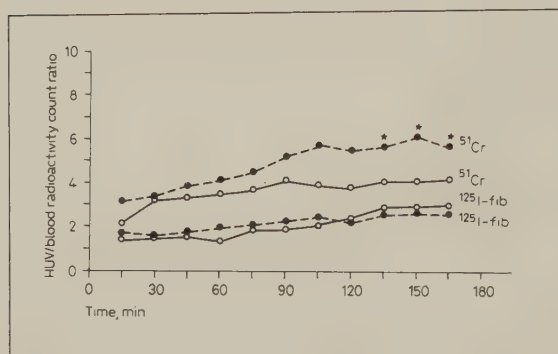


Fig. 2. Uptake of ^{51}Cr and ^{125}I -fibrinogen by heparin-alcohol (○) and heparin without alcohol (●) umbilical veins during 3 h of observation (mean of five experiments). * $p < 0.05$.

Table II. Group II: heparin-alcohol (HAUV) versus heparin without alcohol human umbilical veins (HsAUV)

	TFS, %	Thrombus weight, g	Patency %
HAUV (n = 5)	*88 ± 7	**0.1 ± 0.09	100
HsAUV (n = 5)	53 ± 7	0.6 ± 0.1	100

Results after 3 h of observation. Mean and $\pm$ SE of five experiments.

* $p = 0.01$; ** $p = 0.02$.

grafts (fig. 2). None of the grafts in this group thrombosed. The HAUVs had a significantly greater TFS ($p = 0.01$) and less thrombus weight ($p = 0.02$) than the HsAUVs (table II). The amount of thrombus material was markedly decreased in the HAUVs and HsAUVs but in the latter it covered more luminal surface (fig. 3). As a whole the HAUVs (nine grafts) had a TFS mean of 82% (± 5 SE), a mean thrombus weight of 0.3 g (± 0.1 SE) and 100% patency.

Discussion

Sauvage et al. [15] developed a model to study the thrombogenicity of graft materials for arterial substitutes and found a good correlation with their long-term performance. A modification of their method was used in our study to determine the adhesion and aggregation of platelets in vivo, and the accumulation of fibrinogen on the grafts tested using ^{51}Cr -labelled platelets and ^{125}I -fibrinogen, respectively. We found the NHUVs to be very thrombogenic. When the veins were incubated with heparin followed by storage in ethyl alcohol 50% the thrombogenicity was significantly less compared to nonheparinized grafts and grafts flushed with a heparinized solution. Hufnagel [10] was able to detect 210 mg of labelled heparin/cm² of umbilical vein surface with a concentration of heparin similar to what we used in these experiments. It seems that the binding of heparin to endothelial cells depends on the concentration of the heparin solution and to a lesser degree on the time of incubation [9, 10]. Sauvage et al. [15] compared several



Fig. 3. Luminal surface of a heparin without alcohol human umbilical vein (above) with a small amount of thrombi adhered to the wall. The luminal surface of the heparin-alcohol umbilical vein (below) is free of thrombus material.

prosthetic materials and during 6 h of observation found that the TFS of umbilical veins flushed with a heparinized solution was approximately 13% at a blood flow rate of 50 ml/min.

Even though the mechanism of action is not well understood, heparin seems to exert its anticoagulant effect by facilitating the inhibitory action of antithrombin III. Furthermore, heparinized surfaces have been shown to inhibit platelet adhesion and aggregation [14]. This was also demonstrated in our experiment; nonetheless, the accumulation of fibrinogen was not altered, at least not to the same degree. Maximal platelet adhesion and aggregation occurred between 90 and 120 min and similar results were reported by *Oblath et al.* [13].

In a previous study the fresh umbilical veins were found to adsorb thrombin which was inactivated on contact with antithrombin III. The GAUV did not inactivate thrombin, nor did HsAUV grafts. Yet this function was recovered when they were treated with ethyl alcohol 50%. It has been suggested that

the beneficial effect of the alcohol could be stabilization of the heparin layer possibly by precipitation of the 'structures' responsible for the heparin binding [2]; this nonetheless, remains to be proved.

The present investigation demonstrates (1) that heparinization of the GAUVs before implantation is necessary and (2) that incubation of the GAUVs in heparin followed by storage in ethyl alcohol 50% is more effective in decreasing the acute thrombogenicity than flushing them with a heparinized solution. The HAUV grafts showed a decrease in ^{51}Cr radioactivity, a large TFS, a small amount of thrombus material and 100% patency. It is known that fresh umbilical veins are not suitable for arterial substitutes due to their antigenicity and the development of aneurysms [4].

We are now investigating the effect of heparin-alcohol treatment on the long-term patency and prevention of neointimal fibrous hyperplasia in GAUV grafts in addition to the assessment of the stability of the heparin bond to the luminal surface over time.

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IN VIVO EVALUATION OF THE ACUTE THROMBOGENICITY OF
THE MODIFIED HUMAN UMBILICAL VEIN AND AUTOLOGOUS ARTERY

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ABSTRACT

With the aim to evaluate the acute thrombogenicity, segments of Biografts (Dardik Biograft, Meadox^R), non-heparinized, heparin-alcohol treated or covalently-heparinized, were implanted into the carotid arteries of sheep. Autologous carotid arteries were used as reference grafts. Flow was restricted to 25 ml/min. Accumulation of autologous ³²P-labelled platelets was registered at both anastomotic and mid-graft regions for 240 minutes. At the end of the perfusion period, grafts were removed, opened longitudinally and TFS and thrombus weight determined.

The ex vivo determinations showed a good correlation to results obtained with in vivo registration. In vivo accumulation of platelets was significantly larger in non-heparinized Biografts as compared with heparin-alcohol treated grafts. Covalently-heparinized grafts showed a tendency towards lower radioactivity values, and visual examination post-perfusion revealed areas of "intimal" damage with thrombotic deposits, probably caused by the heparinization procedure.

Autologous arteries showed the largest TFS and the smallest thrombi. Platelet accumulation in autologous arteries decreased almost to reference values after an initial rapid increase.

In conclusion, heparin-alcohol treatment of Biograft reduces the acute thrombogenicity. While covalent heparin bonding seems to act in the same way, the fragility of the graft appears to preclude use of this method. Autologous arteries exhibit excellent antithrombotic characteristics.

INTRODUCTION

The autologous vein is considered the best available graft for use in small diameter artery bypass surgery (1). When not available, human umbilical vein grafts (BIOGRAFT) and expanded polytetrafluoroethylene (PTFE) grafts appear to be the best alternatives (2, 3, 4). In a recent randomized clinical trial, Biografts performed significantly better than PTFE grafts in femoro-popliteal bypass surgery (5).

The procedure for irrigating Biografts immediately prior to implantation includes flushing with heparin, and it has been demonstrated in an experimental canine model, that heparin treatment of the graft surface decreased the acute thrombogenicity, particularly if the luminal surface was incubated with ethyl alcohol after exposure to heparin (6). It thus seems advisable to stabilize the heparin layer. In a similar experimental model in sheep, the performance of PTFE grafts was also significantly improved after covalently bonding heparin to the surface (7).

The aim of the present investigation was to evaluate the acute thrombogenicity of four types of grafts in a sheep model. Three were modifications of the Biograft, untreated, heparin and alcohol treated or covalently-heparinized, and comparison was made with the autologous artery, the ideal vascular graft. The experimental models used included ex vivo evaluation of the luminal surface and assessment of thrombus weights and also in vivo analysis of platelet accumulation along the grafts and anastomoses.

MATERIALS AND METHODS

Operative procedure

Young adult sheep of both sexes, weighing 30-40 kg were used. After premedication with atropin, the animals were anesthetized with sodium thiopental (Pentotal^R), which was then given as a continuous intravenous infusion. They were connected to a respirator with 20% oxygen. The right femoral artery was cannulated for blood sampling and blood pressure recording. The carotid arteries, which have external diameters of 4-5 mm, were exposed through a midline cervical incision, dissected free from surrounding tissue, and tributaries were ligated over a distance of approximately 25 cm. When the autologous artery was used, 6 cm long segments of the carotid arteries were excised and reinserted. When Biografts were used, approximately 4 cm of the host arteries were removed. Transected arteries were flushed with 5 ml of saline solution containing heparin (4 IU/ml) in both proximal and distal directions. The ends of the vessels and grafts were cut at 60° angle and the anastomoses were made end-to-end using running 7-0

polypropylene (Prolene^R, Ethicon). Blood flow was recorded using an electromagnetic flowmeter² (Carolina Medical Electronics Inc., USA) both before and after graft placement. After insertion the grafts were perfused at 25 ml/min for 4 hours, flow being restricted by a distal arterial clamp as described by Sauvage (8). Platelet count, haematocrit and APTT (Automated APTT, General Diagnostics, New Jersey, Ireland) were obtained before and after graft placement and at the end of the experiment.

Evaluation of thrombogenicity

Ex vivo studies of luminal surface and thrombus. After removal, the grafts were opened longitudinally and the luminal surfaces photographed. The thrombus free surface (TFS) was calculated from the photographs using a grid, occluded grafts being considered to have TFS 0 (8, 9). Thrombus weights were also recorded.

In vivo registration of accumulation of ³²P-labelled platelets. Autologous platelets, prepared from a blood sample drawn on the morning of the day of the experiment, were labelled with ³²P using the technique described by Abrahamsen (10) as modified by Wieslander et al (11). After reinfusing the labelled platelets, the radioactivity in the vessels was measured using GM-tubes (Philips type ZP 1410) until steady state. This normally took less than 60 minutes and enabled any platelet trapping to be determined. After inserting the grafts and restricting the flow, the counting-rates over the proximal and distal anastomoses and in the midportion of the grafts were followed for 240 minutes, as were values from a reference point on the proximal carotid artery. One GM-counter was used on each side, this being moved in turn to each counting position. A complete counting cycle normally took about 10 minutes. The counting-rates were recorded for 100 seconds at each position. During the measurements the arteries/grafts were wrapped in thin plastic films to prevent drying, and placed in a specially designed aluminium holder, kept at body temperature by circulating warm water. After removing the clamps, about 25 minutes were normally required for haemostasis, installing the holders and flow reduction. Time 0 represents the time when the flow was restricted.

In addition, the radioactivity in whole blood samples was determined as in earlier studies (7).

Graft material

Human umbilical vein grafts (Dardik Biograft, inner diameter 6 mm) were supplied by Meadox Medicals through ASTRA Meditec, Gothenburg, Sweden.

Non-heparinized Biografts, like the two groups of heparinized grafts, were used in 6 cm lengths and were rinsed with 3000 ml of isotonic saline prior to insertion.

Heparin and alcohol treatment of Biografts. The luminal surfaces of the grafts were incubated with heparin solution (5000 IU/ml) for 12-24 hours, rinsed with saline and the grafts stored in ethyl alcohol for at least 48 hours before use. Immediately before insertion, they were rinsed with 3000 ml of isotonic saline.

Covalent heparin bonding of Biografts was performed by IRD Biomaterial AB (Bromma, Sweden) as described elsewhere (12). This method includes several different steps, some performed at a temperature of 50°C, and results in a stabilized heparin layer with (theoretically) more active groups than are available after conventional covalent bonding. These grafts were also rinsed with 3000 ml saline immediately before insertion.

Autologous arteries. 6 cm long segments of the carotid arteries were excised and gently rinsed with physiologic saline before reinsertion.

Experimental design

A total of 9 autologous carotid arteries, 12 non-heparinized, 8 heparin-alcohol treated and 6 covalently-heparinized Biografts were studied ex vivo with reference to patency, TFS and thrombus weight.

All autologous carotid arteries, heparin-alcohol and covalently-heparinized Biografts and 8 of 12 non-heparinized Biografts were evaluated

in vivo with reference to the continuous accumulation of ^{32}P -labelled platelets. Four non-heparinized Biografts were thus studied only with reference to TFS, thrombus weight and patency after the perfusion period of 4 hours. This was done without using labelled platelets, but with the arteries/grfts placed in the holders used when measuring counting-rates. These four grafts constitute a control group used so as to be able to rule out the possibility that the labelling procedure might be responsible for the poor results obtained with this group of Biografts.

Statistical methods

Values of TFS, thrombus weight and platelet accumulation were compared using the Mann-Whitney U-test for unpaired observations. To evaluate the frequency of occlusion, Fisher's exact test was used. Differences were considered significant when $p < 0.05$.

RESULTS

No changes in platelet count, haematocrit or APTT were observed in any of the experiments. The radioactivity in whole blood samples showed a constant slow decrease throughout the measurements, the residual activity at the end of each experiment being about 70 % of its initial value. The blood pressure recordings during the experiments revealed no gross alterations.

Ex vivo studies

The results for thrombus weight, TFS, patency and time until occlusion for the different groups are summarized in Table I. The luminal surfaces of all patent Biografts were covered by a thin thrombotic film, giving a TFS of 0 for all Biografts. However, inspection of the luminal surfaces revealed obvious differences. In the untreated grafts, the thrombi were firmly attached to the entire graft surface, whereas in heparin-alcohol grafts, they were attached only at the anastomoses. The thrombotic films in covalently-heparinized grafts were also attached to the anastomoses, but, in addition, adhered firmly to several macroscopic visible "intimal" tears (Fig. 1).

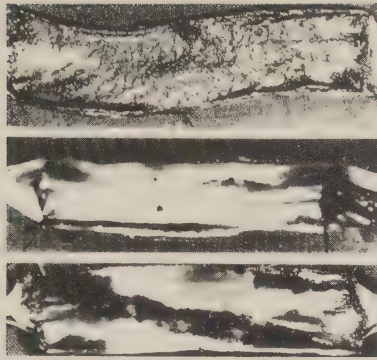


Fig. 1 Photographs of luminal surfaces of non-heparinized (upper), heparin-alcohol treated (middle) and covalently-heparinized (below) Biografts after removal of thrombotic material, showing differences regarding adherence of thrombus.

TABLE I

Patency, TFS, thrombus weight and time until occlusion for different groups of Biograft and autologous artery.

	n	TFS		Thrombus weight mg ($M \pm SEM$)	Patency		Time until occlusion minutes (median and range)
		%			n	%	
Untreated Biograft	12	0		646 $\pm$ 84	1	8	45 (15-135)
Covalently heparinized Biograft	6	0		415 $\pm$ 69	3	50	45 (30-90)
Heparin and alcohol treated Biograft	8	0		212 $\pm$ 32	6	75	110 (105-120)
Autologous artery	9	98.4 $\pm$ 1.0	0.33 (0-3)		9	100	-

Thrombus weights were lower and patency-rates higher for heparin-alcohol treated grafts than for untreated grafts ($p < 0.01$). No differences were observed when comparing either untreated grafts or heparin and alcohol treated grafts to covalently-heparinized ones. TFS and thrombus weights were significantly smaller for the autologous artery than for all types of Biografts.

The thrombus weights were lower in the heparin and alcohol treated group as compared to the non-heparinized grafts ($p < 0.01$), but there was no statistically significant difference between the two methods of heparinization or between covalently-heparinized and non-heparinized grafts. The patency of non-heparinized Biografts was 8 % (1/12), increasing to 75 % (6/8) after heparin and alcohol treatment ($p < 0.01$). Covalently-heparinized grafts were patent in 50 % of the cases (3/6), which is statistically not different, from either the non-heparinized, or the heparin and alcohol treated group. The median time from restriction of flow to occlusion was longer for heparin-alcohol treated grafts, but the ranges of the other groups were broad, and no statistically significant difference was obtained. Patency for autologous arteries was 100 % and the TFS was 98 %, only in one case was a significant area of thrombus covered surface observed. The thrombus in this autologous artery weighed 3 mg. Thus, autologous arteries performed better than all types of Biografts as regards TFS and thrombus weight.

In vivo studies

Radioactivity measurements at the reference points showed a constant, slow decrease to about 70 % of the initial value at the end of the experiment. This paralleled the decrease of activity in whole blood, the residual activity in blood samples also being about 70 % at 4 hours.

After restoring flow through the grafts, a rapid increase of radioactivity was observed in all cases. Both non-heparinized and heparin-alcohol treated Biografts showed essentially uniform distributions of radioactivity along their entire lengths, as is shown in Figures 2 and 3. Covalently-heparinized grafts differed from this pattern, the mid-graft region exhibiting lower activities (Fig. 4). However, no statistically significant differences were encountered in any of the groups. The activities in non-heparinized grafts at the proximal anastomoses and midgraft regions reached maxima 45-60 minutes after restricting the flow, whereas distal anastomoses reached their peak activity after 90 minutes. Following this the activities for all positions decreased slowly throughout the remainder of the period of observation. On the other hand, both groups of heparinized Biografts showed a more plateau-like behaviour, the radioactivity of covalently-heparinized grafts actually showing a slow increase throughout the measurement period.

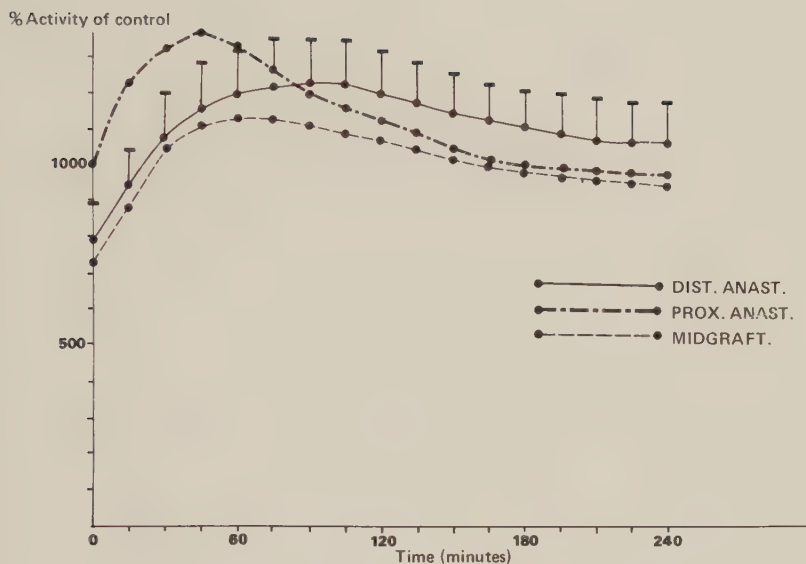


Fig. 2 ^{32}P -platelet accumulation in the different regions of non-heparinized Biografts. (n=8; Mean, SEM is indicated for distal anastomosis).

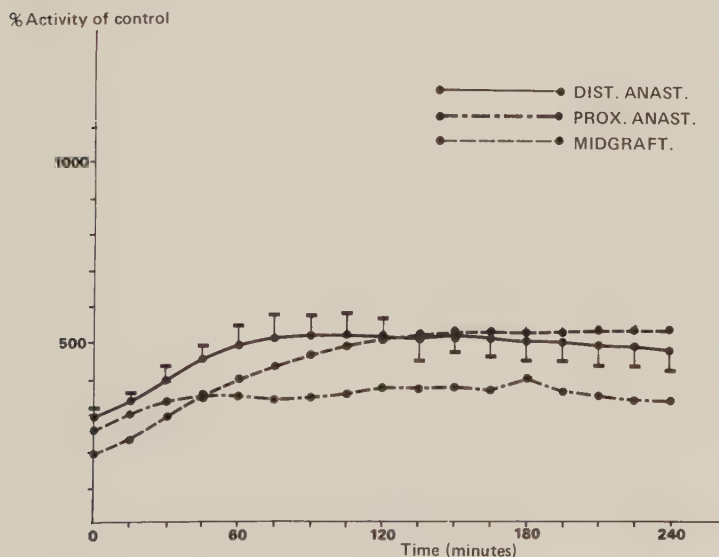


Fig. 3 ^{32}P -platelet accumulation in the different regions of heparin and alcohol treated Biografts. (n=8; Mean, SEM is indicated for distal anastomosis).

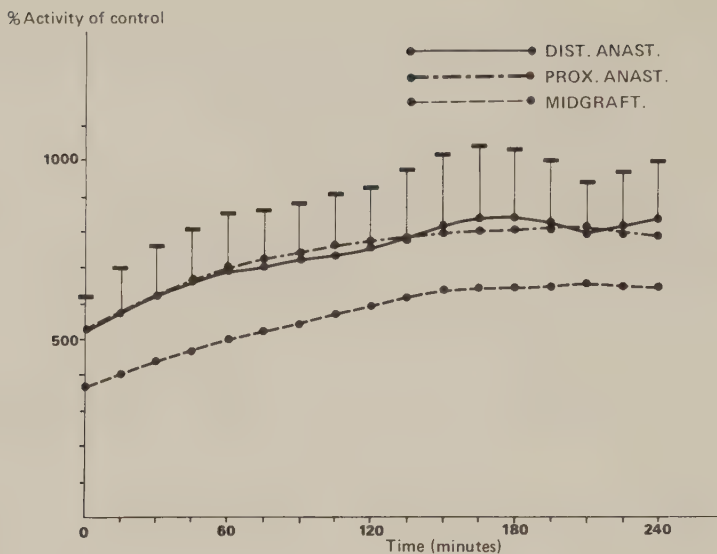


Fig. 4 ^{32}P -platelet accumulation in the different regions of covalently-heparinized Biografts. (n=6; Mean, SEM is indicated for distal anastomosis).

Comparing different methods of heparinizing Biografts (Fig. 5), the activities of heparin-alcohol treated grafts were lower at all times, but the differences were never statistically significant. Compared to non-heparinized grafts, the heparin-alcohol treatment significantly reduced the activity in all regions at all times. Covalently-heparinized grafts only exhibited lower activities at the distal anastomosis between 15 and 75 minutes and in the midgraft position between 15'-45' and 60'-135'. The four non-heparinized Biografts which were perfused without using labelled platelets, did not differ from the other non-heparinized grafts as to TFS or thrombus weight, all occluding early.

Autologous arteries showed higher values of radioactivity over the distal anastomoses, while the midgraft activity paralleled that of the proximal anastomoses. Autologous arteries reached a maximum 15 minutes after restricting the flow and thereafter showed a steady decrease (Fig. 6).

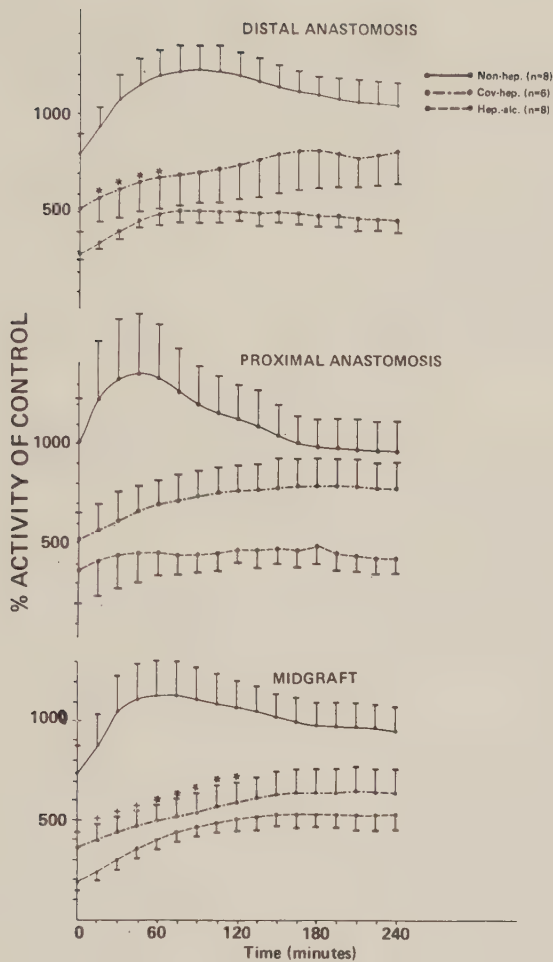


Fig. 5 Comparison of ^{32}P -platelet accumulation in the different regions of non-heparinized (non-hep), covalently-heparinized (cov-hep) and heparin and alcohol treated (hep-alc) Biografts ($M \pm \text{SEM}$).

Values for hep-alc Biografts significantly lower than for non-hep Biografts at all regions during the whole perfusion period ($p < 0.02$ -- $p < 0.01$).

* and + indicate significantly lower values for cov-hep than non-hep Biografts ($p < 0.05$ and $p < 0.02$ resp.).

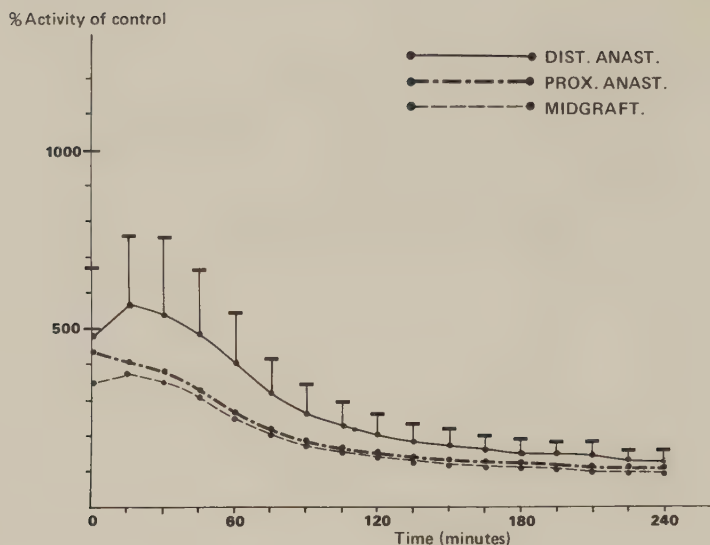


Fig. 6 ^{32}P -platelet accumulation in the different regions of autologous arteries. (n=9; Mean, SEM is indicated for distal anastomosis).

In 8 cases the autologous arteries showed rather similar values of radioactivity in all regions throughout the entire measurement periods. The 9th case exhibited higher activities than the rest, at least in the first half of the experiment. This was the graft mentioned above as being the only one exhibiting a significant thrombus covered surface. On opening it, there was observed an area of endothelial damage, covered by thrombotic material. The behaviour of this artery thus explains the high values for SEM encountered in this group.

After excising but before photographing, as a check on the overall performance of the method, most grafts were scanned using a simple slit-scanning device, with a linear resolution of about 6 mm at the graft, considerably less than the approximately 30 mm resolution in the main runs. The results of these determinations were in very good agreement with those obtained in the main experiments. A more detailed presentation of the experimental techniques used will be given elsewhere (13).

DISCUSSION

Two methods to evaluate acute thrombogenicity have been used in the present investigation, *ex vivo* determination of thrombus weight and TFS as well as patency recordings, and *in vivo* registration of platelet accumulation. The *ex vivo* determinations give exact measures of the size and extent of the thrombus and the visual examination enables an evaluation of the adherence of the thrombus to the luminal surface. However, these determinations give no dynamic information about events taking place during the perfusion period, e.g. transient platelet accumulation.

Labelling platelets with ^{51}Cr has been widely used for a long time (10). However, the gamma radiation from this isotope on the level of a vessel is difficult to register in an *in vivo* model, since the background activity is high, and thick lead shielding has to be used. Despite this, ^{51}Cr -labelled platelets could be used in a previous study in which the total activities of Biografts were recorded (6). Since one of the aims of the present study was to evaluate the spatial distribution of platelets along the grafts, the β -emitting isotope ^{32}P was chosen as being more suitable. Although the efficiency of platelet labelling is lower than for ^{51}Cr , this is more than compensated for by the ease with which the background radiation from all parts of the body other than that being studied can be removed by simple shielding. Small numbers of events thus become significant, and it is possible to follow changes in platelet accumulation over intervals of a few seconds. In addition, it has been shown by other authors (14) that platelets are not damaged by the labelling procedure, do not release their activity to any significant degree and that, in the rabbit, the platelet activity follows the whole-blood activity. These findings are in agreement with several years experience at this laboratory using autologous platelets in sheep (7) and homologous platelets in rabbits (11).

β -particles, even those from a high-energy source such as ^{32}P , have rather short ranges and are rapidly degraded when passing through tissue. For this reason, the values of activity recorded do not reflect the average behaviour of the vessel but are instead weighted,

being strongly biased towards the points closest to the detector. However, as mentioned above, after excision most grafts were scanned in the collapsed state, and the results of these measurements were essentially identical to those obtained in vivo. The results of visual inspection of opened grafts were also in good agreement with the GM-counter data. We thus consider that the data obtained reflect what is happening in the vessel as a whole. These matters will be more thoroughly discussed elsewhere (13).

The present in vivo model is a modification after Sauvage (8), who used dogs and perfused grafts for six hours. He defined the thrombotic threshold velocity (TTV) of a particular graft as the flow velocity at which the average of the TFS scores fell to below 50 %. The value for Biograft was found to be 18.5 cm/second, corresponding to a flow of 150 ml/min in a vessel with an inner diameter of 4 mm. The flow of 25 ml/min used in the present study thus lies below the TTV of Biograft. All Biografts in this study were covered with thin thrombotic films, i.e. even patent grafts had TFS 0. This finding probably reflects the experimental conditions with a flow-rate being slower than the TTV and also with a slight lumen discrepancy between graft and host artery, particularly when there was a slight spasm in the latter. However, there was a marked macroscopic difference between heparinized and non-heparinized grafts. In non-heparinized grafts thrombi were firmly attached to the entire surface, whereas in heparin and alcohol treated grafts the thrombotic material was attached to the anastomoses, but adhered only loosely to the luminal surface. Covalently-heparinized grafts showed, in addition, several intimal "tears" with attached thrombotic material. This suggests that this method of heparinization, which includes several different stages, may be traumatizing.

In the study by Sauvage, Biografts used were rinsed immediately before the operation in 5000 ml of Ringer's lactate, with heparin 10000 IU added to each 1000 ml aliquot. This procedure, however, is not in accordance with the prescription of the manufacturer, which involves as a final step flushing the graft with 30000 IU of heparin solution (5000 IU/ml). In our previous study using ⁵¹Cr-labelled platelets in dogs, this treatment was shown to decrease the thrombogenicity, this being further accentuated by alcohol treatment (6). These results

are confirmed by the present investigation. Moreover, from the radioactivity measurements, the results for the covalently-heparinized grafts indicate that this method may also improve the antithrombotic characteristics of Biografts.

In a study comparing platelet accumulation in autologous veins and Biografts in a canine model, Roedersheimer et al (15) found Biograft to be inferior to autologous vein due to its poor handling characteristics and to the fact that platelets exhibited a pronounced tendency to adhere to the graft surface. Biograft is a musculo-collagenous tube comprising several distinct layers of collagen and muscle, which, when handled improperly, may split up and cause flaps and subintimal dissection and/or thrombosis. When handled in accordance with the technical instructions (16, 17), which include avoiding passing the needle through the wall from outside, these difficulties can be overcome. For instance, our quite extensive experience of this graft includes only one case of subintimal dissection.

In his study Sauvage found the TTV for autologous artery to be almost 0 (8). This is confirmed by our results, indicating that the autologous artery is the superior arterial graft. This suggestion is further supported by the behaviour of the ninth artery, which, despite the evidence of an extremely large platelet accumulation, due to a localized endothelial damage, did not occlude.

On basis of the results of the present investigation, the following conclusions can be drawn:

The in vivo method presented here, using ^{32}P -labelled autologous platelets, offers a good alternative in studying the performance of different vascular graft materials and is capable of distinguishing among the effects of different surface modifications, e.g. heparinization procedures. In addition, there was a good agreement between results from this method and ex vivo data.

Non-heparinized human umbilical vein grafts (Dardik Biograft) possess a high acute thrombogenicity, which can, however be markedly reduced by heparin and alcohol treatment. While covalent heparin bonding seems

to act in the same way, the fragility of the graft appears to preclude use of this method in situations of clinical interest.

The autologous artery has a very low acute thrombogenicity, as shown by both in vivo and ex vivo determinations.

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Reduced thrombogenic characteristics of expanded polytetrafluoroethylene and polyurethane arterial grafts after heparin bonding

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The effect of bonding of heparin, via a glutaraldehyde-stabilized ionic complex, on the early thrombogenicity of polyurethane (PU) and expanded polytetrafluoroethylene (PTFE) (Gore-tex) as well as covalent bonding of heparin on PTFE was studied in vivo. Grafts 6 cm long and 4 mm in diameter were placed in the carotid arteries of sheep and perfused for 4 hours at 25 ml/min in order to accelerate thrombus formation. The thrombogenicity was determined by calculation of the percent of the luminal surface free of thrombus and patency. In addition, ^{32}P -labelled platelet accumulation was determined in some of the grafts. The stabilized ionic bonding of heparin significantly reduced the early thrombogenicity of PU but had little effect on PTFE grafts; but the thrombogenicity of the latter was markedly decreased following covalent bonding of heparin. A regional distribution of platelet accumulation was found with the distal anastomoses showing the highest platelet deposition. By choice of an appropriate method of heparinization, a significant reduction of the thrombogenicity of PU and PTFE grafts was achieved.

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POOR PERFORMANCE of small-diameter vascular substitutes (<6 mm) is a frequent occurrence in low-flow, high-resistance locations.² The thrombogenicity of the graft material is a significant factor incriminated in early failures.^{6,10,26} Although some improvement has been achieved by modification of the physical characteristics of the materials (i.e., porosity, compliance, durability),^{17,29} it appears that an adjuvant antithrombotic therapy or binding of a pharmacologic agent to the surface is necessary in order to improve patency rates.^{2,4,19}

Platelets are major determinants of thrombus forma-

tion in the arterial system;^{22,24} however, platelet-compatible surfaces may still be thrombogenic by activation of the coagulation enzymes.¹³ Larsson et al.¹³ demonstrated an increase in platelet and plasma coagulation compatibility in heparinized polyethylene tubes.

The purpose of this work was to study the effect of heparin bonding on the early thrombogenicity of hydrophobic polymers used as arterial substitutes.

METHODS AND MATERIALS

Animal model. Adult sheep of either sex with an average weight of 44 kg were studied. They were fasted for 18 hours before the experiment. After premedication with atropine, the animals were anesthetized with sodium pentobarbital and kept on a respirator with 20% oxygen. The carotid arteries that had an external diameter of 5 to 6 mm were dissected out through a midline cervical incision. The transected artery was

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flushed with 5 ml of saline solution containing heparin (4 IU/ml). Grafts 6 cm long and 4 mm in diameter were interposed in the carotid arteries after removal of 4 cm of the host vessel. The ends of the vessel and the graft were beveled at a 45-degree angle, and the anastomoses were made end-to-end with running 7-0 polypropylene sutures. With an electromagnetic flowmeter, the blood flow was recorded before and after graft placement. Grafts were perfused at 25 ml/min for 4 hours by placement of a distal arterial occluder in order to accelerate thrombus formation and to create a condition of high resistance and low flow, as frequently occurs in patients with peripheral arterial disease.²⁶ The blood flow chosen in these experiments is approximately one sixth of the normal carotid blood flow in sheep. In a few experiments (data not included) a blood flow of 50 ml/min was selected; however, the lack of thrombus formation on the heparinized polyurethane (PU) surface led us to restrict the flow even further. Upon removal, grafts were opened longitudinally and the luminal surface was photographed. The thrombus-free surface (TFS) was calculated with use of a grid; occluded grafts were considered to have a TFS of 0.4%.

Platelet counts and activated partial thromboplastin time (APTTs) were obtained before, immediately after, and 30 minutes after graft placement.

Labelling of platelets and registration of radioactivity. Autologous platelets were labelled with ³²P and reinfused before graft placement.^{1,30} The carotid artery was placed in a rectangular aluminum shield and the radioactivity was recorded with Geiger Müller (GM) tubes (Philips type 18505). The shields were kept at body temperature by circulation of warm water inside the device.

The radioactivity pulses were recorded for 100 seconds at 15-minute intervals (Selectronix Scaler-Timer model 47-21) over a period of at least 3 hours in addition to continuous recording on a pen recorder. Two independent counting channels were used for simultaneous recording of both carotid arteries. After the injection of tagged platelets the radioactivity was recorded for approximately 1 hour until it reached a steady-state. Following graft placement, the activity was determined over the proximal and distal anastomoses and also over the midportion of the graft. Recordings from the proximal carotid arteries were used as the reference points. After the clamps were removed, there was a lapse of approximately 15 minutes for hemostasis and installation of the lead shields. Time 0 represented the time when the blood flow was restricted.

In addition, the radioactivity in the whole blood and blood fractions—platelets, plasma and corpuscular fractions—was determined 30 minutes after infusion of tagged platelets and then at hourly intervals for 4 to 6 hours. The radioactivity was measured in a β -ray analyzer (LKB-Wallac 81000 liquid scintillation counter, Finland) and the results were quench corrected.²³

Heparinization of material. Heparin coating of the graft materials was performed by IRD Biomaterial AB (Bromma, Sweden).

Stabilized ionic bonding of heparin. Polyurethane (Cil-Monothane Elastomer, Grade Hardness Shore A-30) and expanded polytetrafluoroethylene (PTFE) (Gore-tex) grafts were treated with a solution of polyethyleneimine and glutaraldehyde for 5 minutes at room temperature. This was followed by treatment with a heparin solution (porcine-mucosal heparin; USP, 141 mg/kg; Kabi Vitrum, Stockholm) (0.1 mg/ml) at 50° C for 5 minutes. This treatment renders the surface negatively charged. Grafts were then treated with a solution containing colloidal particles of heparin and hexadecylaminehydrochloride for 2 minutes at 50° C, followed by treatment with a heparin solution (0.1 mg/ml). The last two steps were repeated three times. The ionic heparin complex was finally stabilized with glutaraldehyde and rinsed with ultrapure water.

Covalent bonding of heparin. Grafts were treated with a solution of polyethyleneimine and glutaraldehyde as described above. Subsequently, the surfaces were treated with dextran sulfate (0.1 mg/ml) at 50° C for 5 minutes, leaving the surface negatively charged. This is again followed by treatment with polyethyleneimine; heparin with an active aldehyde group in the terminal end of the molecule (obtained by partial degradation with nitrous acid) was bonded to the aminated surface. The reaction was performed in a water solution containing 0.4 mg/ml heparin and 0.04 mg/ml of cyanoborohydride at a pH of 3.8 for 2 hours at 50° C. These two procedures are described in detail elsewhere.^{12,13}

Experimental design

Polyurethane grafts. Stabilized ionic bonding of heparin was performed on 11 PU grafts (Hep-glut PU) and compared to ten nonheparinized polyurethane grafts with regard to TFS and patency. In five of these experiments the platelet deposition was studied in Hep-glut PU and compared to PU grafts placed in the contralateral carotid arteries.

Polytetrafluoroethylene grafts. Stabilized ionic bonding of heparin was performed in eight (Hep-glut

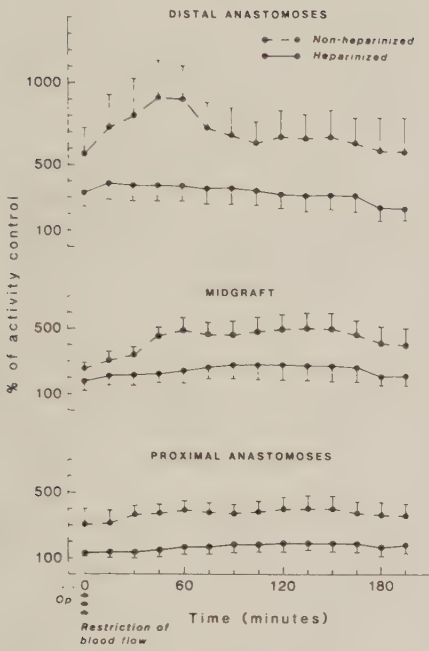


Fig. 1. Effect of stabilized ionic bonding of heparin to PU grafts on accumulation of ^{32}P -labelled platelets compared to untreated PU grafts in the proximal and distal anastomoses as well as midgraft ($n = 5$) (mean $\pm$ SEM).

PTFE) and covalent bonding of heparin in eight PTFE grafts (Cov-hep PTFE). The results were then compared to those with five untreated PTFE grafts. The platelet accumulation along the graft was investigated in six Cov-hep PTFE grafts.

Statistical analysis. The results of TFS were analyzed by Mann-Whitney U test. The radioactivity values were rendered to a more normal distribution by square root transformation, and the significance of difference was calculated by paired Student's t test.

RESULTS

The mean arterial blood flow was 145 ± 20 ml/min, and there was no significant variation in the blood flow before and after graft placement. No change of the platelet counts or APTTs was observed.

Polyurethane grafts. The results of the PU materials are summarized in Table I. Heparin bonding increased the TFS $57 \pm 10\%$ in the Hep-glut PU grafts and $27 \pm 8\%$ in the PU grafts, and the difference was statistically significant ($P = 0.0207$). Patency also improved after heparin bonding, and only one of 11

Table I. Comparison between heparinized and untreated PU grafts

Material	n	TFS (%)	No. thromboses
Hep-glut PU	11	$57 \pm 10^*$	1
PU	10	27 ± 8	3

* $P = 0.0207$

Hep-glut PU grafts thrombosed as compared to three of ten PU grafts. In the former, the thrombus did not adhere to the luminal surface but to the anastomotic sites.

The radioactivity in the platelet-rich plasma (PRP) fraction ranged from 75% to 88% of the whole blood activity, and no significant change was noticed throughout the experiments.

Compared to the PU grafts, heparin bonding significantly reduced platelet accumulation (Fig. 1). Compared to the reference point values, an increase in the radioactivity from 100% to 300% occurred in the distal anastomoses of the Hep-glut PU grafts by the time the first measurement was performed. In contrast, the ^{32}P activity had increased to almost 600% in the distal anastomoses of PU grafts during the same period and reached 900% 45 to 60 minutes later. Thereafter, a moderate decrease in the ^{32}P activity took place and leveled off during the rest of the period of observation. The difference was statistically significant ($P < 0.05$) except in the first determinations. The difference in the ^{32}P -labelled platelet accumulation between the two types of grafts was of greater significance in the midgraft and proximal anastomoses; in the midgraft, the difference was not statistically significant during the first three determinations, but thenceforth the P values ranged from 0.025 to <0.005 . In the proximal anastomoses the P values ranged from 0.025 to <0.005 throughout the experiment.

The deposition of tagged platelets in the distal anastomoses was higher than in the proximal anastomoses of the Hep-glut and PU grafts.

Polytetrafluoroethylene grafts. Stabilized ionic bonding of heparin improved the patency rate from 30% to 60%, although the TFS remained unchanged. However, the TFS of PTFE was significantly reduced after covalent bonding of heparin, and only one of eight grafts clotted (Table II).

^{32}P -labelled platelet accumulation in the Cov-hep PTFE grafts was similar to that of Hep-glut PU grafts. A regional platelet distribution was also observed in the Cov-hep PTFE grafts, and the area of the distal anastomoses showed the highest activity

Table II. Results of covalent and stabilized ionic bonding of heparin onto PTFE grafts compared to untreated PTFE grafts

Material	n	TFS (%)	No. thromboses (%)
Cov-Hep PTFE	8	57 ± 11*	1 (13)
Hep-glut PTFE	7	23 ± 12	2 (30)
PTFE	5	27 ± 16	3 (60)

* $P = 0.027$ versus Hep-glut PTFE; $P = 0.033$ versus PTFE.

throughout the experiments (Fig. 2). During the first half of the experiments the platelet activity was higher in the proximal anastomosis than in the midgraft, but in the second half the activity equalized in these two regions. During the first 2 hours, the ^{32}P -labelled platelet accumulation was significantly greater in the distal anastomoses ($P < 0.05$ to < 0.025) and midgraft ($P = 0.025$ to 0.005).

DISCUSSION

Expanded PTFE grafts are frequently used in patients with lower limb ischemia when autogenous saphenous veins are not available or not of good quality.^{3,28} Nevertheless, this material has been shown to be thrombogenic in clinical and experimental practice.^{4,6} In a recent review, Callow² pointed out the fact that in low-flow, high-resistance locations, small-diameter prostheses (< 6 mm) do not perform well.

In part the limited success with artificial vascular prostheses is due to a lack of an adequate in vivo test that could predict graft patency. Sauvage et al.²⁶ developed an in vivo method to study the thrombogenicity of graft materials in dogs that was modified for use in sheep.⁴ The dog does not appear to be a good animal model for testing biomaterials and cardiovascular devices (NIH publication 80-2185), mainly because of its active hemostatic and fibrinolytic systems, its very reactive vessels that lead to occlusion, and the reactivity of canine platelets to heparin. Sheep have been reported to have coagulation parameters similar to those of humans.²⁷ With this method modified to measure platelet deposition, we have studied the effect of heparin bonding on the thrombogenicity of PU materials and to commercially available PTFE. ^{32}P instead of ^{51}Cr was used to label platelets, since it was easier to isolate the vessel graft activity because of the short range of the β -particles as compared to α -radiation. The activity ranged from 75% to 88% in the PRP fraction and remained steady during the course of the experiments. We found that the thrombus thresh-

% activity of control

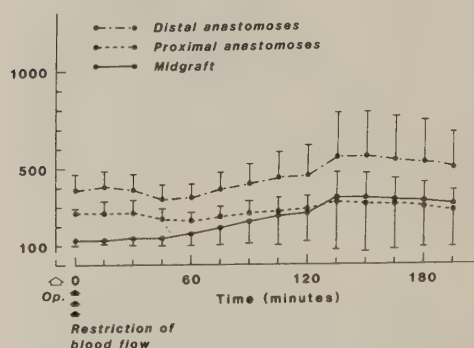


Fig. 2. ^{32}P -labelled platelet accumulation in the proximal and distal anastomoses and middle of covalently heparinized PTFE grafts ($n = 6$) (mean $\pm$ SEM).

old velocity, (as described by Sauvage) for the PTFE grafts was 50 ml/min;⁴ therefore, restriction of the flow to 25 ml/min should cause thrombosis or heavy thrombus deposition.

Several reports have demonstrated that heparin bonding to a polymer increases platelet compatibility,^{11,13,21} but on the other hand, platelet-compatible surfaces may still be thrombogenic by activation of the coagulation enzymes in the cascade.¹³ Larsson et al.¹³ showed that hydrophobic polymer (polyethylene) furnished with a stabilized heparin ionic complex was platelet and plasma coagulation compatible in in vitro and in vivo tests. Stabilized ionic bonding of heparin reduced the early thrombogenicity of PU but had little effect on PTFE grafts. The different results are probably related to variations in the physicochemical structures of these two materials. Being less hydrophobic than the PTFE, the PU surface probably provided a better substrate for bonding of the heparin-hexadecylamine complex. On the other hand, covalent bonding of heparin did significantly decrease the thrombogenicity of PTFE grafts.

We have observed that the anastomoses create a strong stimulus for thrombus formation probably by exposure of the subendothelial structures to the circulating blood.⁸ Kenny et al.¹⁰ reported 100% patency in Gore-tex grafts placed in the carotid arteries of dogs 1 week after implantation, but patency dropped to 16% 2 weeks later. They suggested that factors at the anastomoses are responsible for the eventual closure, particularly in the proximal anastomoses.

Decreased platelet deposition was demonstrated in the heparinized PU material compared to the untreated

PU. Similar findings have been reported with other heparinized polymers.^{11,13,21} Reports in the literature conflict in regard to the effect of heparin on platelets.^{20,25} Besides the differences in method, species variations, and heterogeneity of heparin molecule, it appears that unbound heparin may enhance platelet activity,^{20,25} whereas bound heparin increases platelet compatibility.^{11,13,21} Although heparinized surfaces do not prevent platelet adhesion completely, they certainly reduce platelet deposition significantly when compared to nonheparinized surfaces. It is important to emphasize that platelets are important in the healing process; therefore, and in simplistic terms, platelet adhesion should be enough to stimulate healing and not excessive to induce thrombus formation. Healing with the formation of a neointima will make the surface less thrombogenic.⁹

A higher level of platelet activity was found in the area of the distal anastomoses compared to the midgraft and proximal anastomoses and similar results have been reported.⁵ It is possible that flow turbulence at the proximal anastomosis may activate platelets by disruption of red blood cells (RBCs) with the release of adenosine diphosphate, (ADP), and platelets will then adhere just proximal to the distal anastomoses, another zone of turbulence. In the proximal anastomosis, the production of prostaglandin I_2 by the artery just proximal to the graft may reduce the platelet adhesion whereas in the distal anastomosis the foreign material does not for certain have the same protective effect.

It is interesting to point out that in relation to platelet activity a quiescent laser-induced injury downstream is reactivated by inflicting a fresh endothelial injury upstream following the release of ADP from injured RBCs.¹⁸ Therefore, a similar phenomenon may occur in the presence of a vascular prosthesis.

Ionic bonding of heparin has the disadvantage of heparin loss, but this has been prevented by treatment with glutaraldehyde. After an initial loss of approximately 12%, no further loss was noted for 7 days in experiments *in vivo*.¹⁴ Covalently bonded heparin is stable but appears to be less platelet compatible,¹⁵ which may be secondary to bonding of active sites resulting in decreased heparin activity. The covalent bonding used in these experiments is a new method that bonds only the end of the heparin molecule to the surface. This type of conformation allows most of the molecule to be free with the advantage of leaving more active sites available and because of the covalent type of bonding heparin loss is not to be expected; nevertheless, experiments to determine the long-term stability of the heparin bonding are being conducted. Although

it is too early to say, this method of heparinization seems promising.

CONCLUSIONS

Stabilized ionic bonding of heparin reduced the early thrombogenicity of PU but not of PTFE grafts. Covalent bonding of heparin reduced the thrombogenicity of PTFE grafts. Regional platelet deposition occurred throughout the graft, with the distal anastomotic area exhibiting the highest level of platelet activity.

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Se incorporo heparina en injertos de PTFE (politetrafluoretileno) (Gore-tex) y de poliuretano (PU) por medio de un complejo iónico de glutaraldehído estabilizado. Asimismo, se incorporó heparina en injertos de PTFE utilizando el método de incorporación covalente.† Se estudiaron in vivo los efectos que tuvieron estos métodos de incorporación de heparina sobre la trombogenicidad de los injertos. En las carótidas de corderos se colocaron injertos de 6cm de longitud por 4mm de diámetro. Los injertos fueron perfundidos durante 4 horas a razón de 25 ml/min con objeto de acelerar la trombosis. La trombogenicidad se calculó midiendo el porcentaje de superficie de la luz del injerto que se encontró libre de trombos, y la permeabilidad del injerto. Además, en algunos de los injertos se determinó la acumulación de plaquetas marcadas con P³². La incorporación iónica-establecida de heparina disminuyó notablemente la trombogenicidad temprana en PU pero tuvo poco efecto sobre los injertos de PTFE; la trombogenicidad de estos últimos, sin embargo, disminuyó notablemente con la incorporación covalente de heparina. Se observó una acumulación regional de las plaquetas marcadas, las cuales mostraron una tendencia a depositarse en las anastomosis distales. Mediante la selección adecuada del método de heparinización, se pudo obtener una disminución muy significativa en la trombogenicidad de los injertos PU y PTFE.*

*Bonding- Incorporación de un material en otro. En este caso, heparina.

†Covalent- Incorporación química no iónica de un material en otro.

THROMBOXANE PRODUCTION IN UMBILICAL VEIN GRAFTS

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ABSTRACT

Umbilical vein grafts were placed in the carotid arteries of sheep and removed after 10 or 90 days for determination of *in vitro* production of 6-keto-PGF_{1α} and thromboxane B₂ (TxB₂) in the anastomoses and in the middle of the grafts. At both intervals the grafts produced much more TxB₂ than carotid arteries, but no significant difference in production of 6-keto-PGF_{1α} was found between grafts and carotid arteries. The strongly decreased 6-keto-PGF_{1α}/TxB₂ ratio indicates a low resistance to platelet adhesion and aggregation, which may be a contributory cause of the thrombogenicity of vascular grafts and may possibly be involved in the pathogenesis of neointimal fibrous hyperplasia.

INTRODUCTION

Thrombosis is a common cause of failure of small-diameter vascular prostheses (1). The risk of thrombosis is higher with artificial materials than with autogenous vessels and probably involves several different pathogenetic factors, e.g. a reduced ability of the graft surface to inactivate thrombin (2) and activation of platelets.

Platelet survival is decreased during the first year after vascular grafting (3), suggesting platelet consumption on the graft surface, which may result in thrombosis. One cause of this platelet consumption may be a disturbance in the prostacyclin/thromboxane ratio in the neointima.

In the present investigation we have therefore studied the *in vitro* production of 6-keto-PGF_{1α} and thromboxane B₂ (TxB₂), stable hydrolysis products of prostacyclin (PGI₂)_α and thromboxane A₂, respectively, in vascular grafts inserted in the carotid artery in sheep. A preliminary report on some of the

Key words: thromboxane, 6-keto-PGF_{1α}, vascular grafts

results concerning 6-keto-PGF_{1α} has been published earlier (4).

MATERIAL AND METHODS

Surgical procedure. Mature sheep of either sex with an average weight of 41 kg were used. The operations were carried out under strictly sterile conditions and the animals received 1.2 million units of benzathine penicillin G intramuscularly before and 12 hours after the surgical procedure. The animals were premedicated with atropine, anaesthetized with sodium pentobarbital (Mebumal®) and ventilated by a respirator with 20 % oxygen. The surgical procedure has been described in detail elsewhere (4,5). Briefly, it consisted of placing interposition grafts measuring 6 cm in length after resection of 4 cm of the carotid arteries. The ends of the vessel and the grafts were beveled at an angle of 45° and the anastomoses were made end-to-end with running 6-0 polypropylene sutures. The external diameter of the carotid arteries was 5-6 mm. The blood flow was recorded with an electromagnetic flowmeter (Carolina Medical Electronics, Inc, USA) before and after graft placement and later before removal.

Grafts. Glutaraldehyde-tanned human umbilical vein grafts (Dardik, Bio-graft, Meadox). Half of the grafts were treated with a heparinized solution (5000 IU/ml porcine mucosal heparin, Kabi-Vitrum, Stockholm, Sweden) and subsequently with ethyl alcohol 50 %; the other half were treated with the same heparin solution but not with the alcohol (6). As no significant difference was found between alcohol-treated and non-alcohol-treated grafts with respect to the production of 6-keto-PGF_{1α} and TxB₂ the two groups are combined in the following presentation.

Experimental design. Twelve umbilical vein grafts (6 treated with heparin and alcohol and 6 treated with heparin alone) were removed after 90 days. The grafts were perfused at a normal physiological blood flow. Eleven of the grafts were patent and one was occluded by a thrombus. Three grafts showed small thrombi on their surfaces.

Eight umbilical vein grafts (4 treated with heparin and alcohol and 4 treated with alcohol alone) were removed on the 10th postoperative day. In these grafts the blood flow had been reduced to 50 ml/min at the end of the operation. Six of them were patent and two were occluded by thrombi. Five grafts showed small thrombi on the graft surface.

Harvest of prostheses. The animals were anaesthetized and 200 IU/kg of heparin were given intravenously before the procedure. The prosthesis and approximately 6 cm of the adjacent vessel proximal and distal to it were removed. Patency was determined by measuring the blood flow and by direct observation of bleeding through the transected and distal to the graft. After removal of the periprosthetic tissue, sections from the anastomotic sites including a portion of the artery and a portion of the graft (half and half) and also sections from the mid-graft were tested for production of 6-keto-PGF_{1α} and TxB₂ (FIG.1). Segments from the carotid arteries and from jugular veins served as controls.

Incubation of the grafts. The specimens of luminal tissue surface, which measured 0.5 to 1 cm², were immediately rinsed in ice-cold Ringer's solution with a pH of 7.4. Any gross thrombus material was carefully removed and the specimens were superfused with Ringer's solution. They were then transferred into fresh Ringer's solution and incubated for 15 min. at 37°C. After this they were transferred into another fresh solution and reincubated for a further 15 min. Aliquots of the supernatant were frozen at -70°C until TxB₂ and

6-keto-PGF_{1α} assays were performed.

The production of 6-keto-PGF_{1α} and TxB₂ was determined by radioimmunoassays (RIAs), using labelled antigen and antirabbit antiserum as antibody (6-keto-prostaglandin F_{1α} ¹²⁵I or ³H RIA Kits and thromboxane B₂ ³H RIA Kit, New England Nuclear). Of the TxB₂ antibody, 2 % cross-reacts with PGD₂, 0.1 % with PGE₂ and PGF_{2α}, 0.08 % with 6-keto-PGF_{1α} and less than 0.005 % with PGA₂. Of the 6-keto-PGF_{1α} antibody 2.5 % cross-reacts with PGF_{2α}, 1.9 % with PGE₁, 1.4 % with TxB₂, 1.1 % with PGE₂, 0.8 % with PGF_{1α}, 0.2 % with PGA₁ and PGD₂, 0.05 % with arachidonic acid and 0.04 % with PGA₂. Each sample was assayed in duplicate.

Statistical methods. Student's t-test and Wilcoxon's rank sum test of paired observations were used. Differences were considered significant when $p < 0.05$. The 6-keto-PGF_{1α}/TxB₂ ratios were not normally distributed and therefore the normalizing data transformation $R' = (R + 1)$ was used before calculation of means and variability (7). Variability is expressed as the 95 % confidence of the mean rather than the standard deviation, since the applied data transformation makes the variability measure asymmetrical around the mean.

RESULTS

There were no significant differences between patent grafts, with and without small thrombi, and occluded grafts concerning the production of 6-keto-PGF_{1α} and TxB₂ and these three groups will therefore be treated as one.

The grafts removed after 90 days showed a very high production of TxB₂ in comparison with carotid arteries and jugular veins of the same animals (FIG. 2). The mid-graft portions may represent the ability of the neointima to produce TxB₂. The finding of as much TxB₂ production in the anastomosis as in the mid-graft suggests increased production of TxB₂ also in the arterial parts of the anastomosis, as the samples from the anastomotic area contained as much arterial tissue as graft (FIG. 1). The production of 6-keto-PGF_{1α} was not significantly different from that in the vessels (FIG. 3), although there was a strong tendency towards a lower production of 6-keto-PGF_{1α} in the mid-grafts ($p < 0.054$). The 6-keto-PGF_{1α}/TxB₂ ratios were significantly lower in the grafts than in the carotid arteries (FIG. 4).

The grafts removed after 10 days also showed a high production of TxB₂ compared with the carotid arteries (FIG. 5A), although the TxB₂ production was not as high as in the 90-day grafts. The production of 6-keto-PGF_{1α} in the grafts did not differ significantly from that in the carotid arteries (FIG. 5B) and the 6-keto-PGF_{1α}/TxB₂ ratios were significantly lower in the grafts - the mean ratio and the 95 % confidence of the mean being 78 (20-298) for carotid arteries, 17(8-32) for proximal anastomosis, 6(2-16) for midgraft, and 9(4-19) for distal anastomosis.

Human umbilical vein grafts not used in the animals produced no 6-keto-PGF_{1α} or TxB₂.

DISCUSSION

It has been proposed that decreased synthesis of PGI₂ may be a critical factor in the development of thrombosis, on the basis that generation of PGI₂ may underly the ability of normal vessels to resist platelet adhesion (8). Vascular synthesis of thromboxane is generally believed to be non-existent or of very slight degree and of no physiological or pathological importance. We

have found in earlier studies, however, that in atheromatous arteries the production of TxB_2 is much increased (9) and can even exceed that of 6-keto-PGF $_{1\alpha}$. As TxB_2 is known to induce platelet aggregation, this observation gave us reason to suggest that under certain circumstances increased vascular production of TxB_2 might be contributory to thrombosis.

In the present investigation we found that in vascular grafts, the production of TxB_2 was increased, whereas the production of 6-keto-PGF $_{1\alpha}$ was not significantly different from that in normal arteries. The strongly decreased 6-keto-PGF $_{1\alpha}/\text{TxB}_2$ ratios in the grafts indicate a diminished resistance to platelet adhesion and aggregation and might well be a contributory factor to graft thrombosis. In addition, this mechanism may be involved in the pathogenesis of neointimal fibrous hyperplasia by increasing platelet aggregation and release of platelet-derived growth factor, which may stimulate smooth muscle cell proliferation.

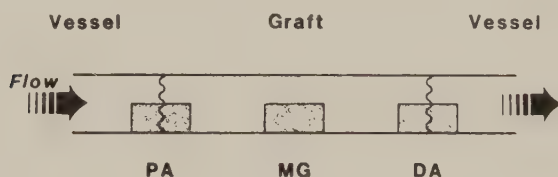


FIG. 1.

Square areas represent the segments submitted for determination of TxB_2 and 6-keto-PGF $_{1\alpha}$.
PA = proximal anastomosis, MG = midgraft, DA = distal anastomosis.

The reason for the production of TxB_2 was not revealed in the present study. Neither is it known which type of cell or cells in the neointima are responsible. Endothelial cells have been reported to produce TxB_2 (10). The endothelial cells underlying the anastomosis are large and pleomorphic (4) and it is possible that these abnormal cells might have an increased ability to produce TxB_2 . Fibroblasts (11), leucocytes (12), smooth muscle cells (13) and platelets (14) have all been shown to produce TxB_2 . However, sheep platelets were reported to produce only minute amounts of TxB_2 (15). That the production of TxB_2 was stronger in the 90-day than in the 10-day grafts may indicate that inflammatory cells are not of major importance as the source of TxB_2 in the former grafts.

In the 10-day grafts inflammatory cells may play a greater role. Leucocytes firmly adherent to the graft may be partly responsible, as in prelimi-

nary experiments with synthetic grafts removed after 4 hours some of the mid-grafts produced both TxB_2 and 6-keto- $\text{PGF}_{1\alpha}$. This production should be due to blood cells stuck to the graft surface. Leucocytes are more probably involved than platelets, also since the latter have been reported not to produce

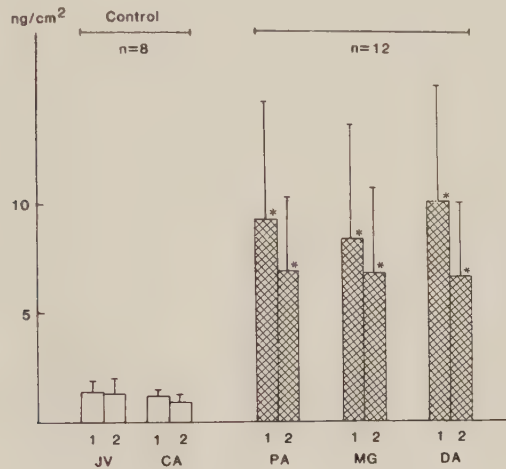


FIG. 2.

TxB_2 synthesis from anastomotic sites and midgraft of prosthesis placed in the carotid arteries of sheep and removed 90 days later. Mean $\pm$ S.D.

JV = jugular vein, CA = carotid artery, 1 = first 15 min. incubation period, 2 = second 15 min. incubation period
x = significantly different from CA.

6-keto- $\text{PGF}_{1\alpha}$ (16). Of the leucocytes, monocytes are known to stick more firmly to foreign surfaces than granulocytes and lymphocytes. Monocytes produce TxB_2 as well as 6-keto- $\text{PGF}_{1\alpha}$ (17), and macrophages have been reported to contain much more arachidonic acid than other cells (18).

Trauma and stress at the arterial part of the anastomoses may be one cause of the increased production of TxB_2 in this region, as balloon catheter trauma gives rise to an increased production of TxB_2 in human umbilical veins (19).

Our observation of production of 6-keto- $\text{PGF}_{1\alpha}$ in the neointima confirms the finding by Clagett et al (20), that the neointima produced 6-keto- $\text{PGF}_{1\alpha}$, although not as much as native arterial tissue.

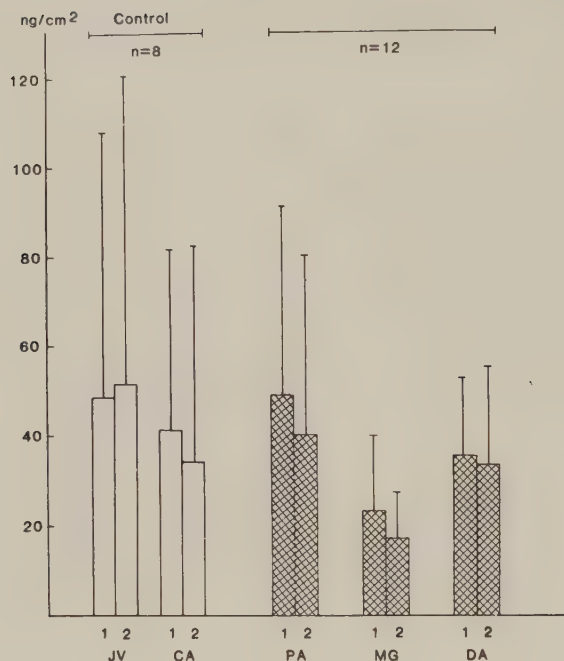


FIG. 3.

6-keto-PGF_{1α} synthesis in grafts removed after 90 days.
For code, see Figs. 1 and 2.

If the lower 6-keto-PGF_{1α}/TxB₂ ratios in the grafts, compared with normal arteries, observed in the present investigation implies a lower resistance to graft thrombosis, then prophylactic treatment of such thrombosis should include inhibition of the TxB₂ production in the neointima and anastomoses. We have found that a thromboxane synthesis inhibitor (UK 37,248, Dazoxiben[®]) prevents production of TxB₂ in the vessel wall but leaves the production of 6-keto-PGF unchanged (21) and that another thromboxane inhibitor, (ibuprofen, Motrin[®]) reduces the thrombogenicity of PTFE grafts (5).

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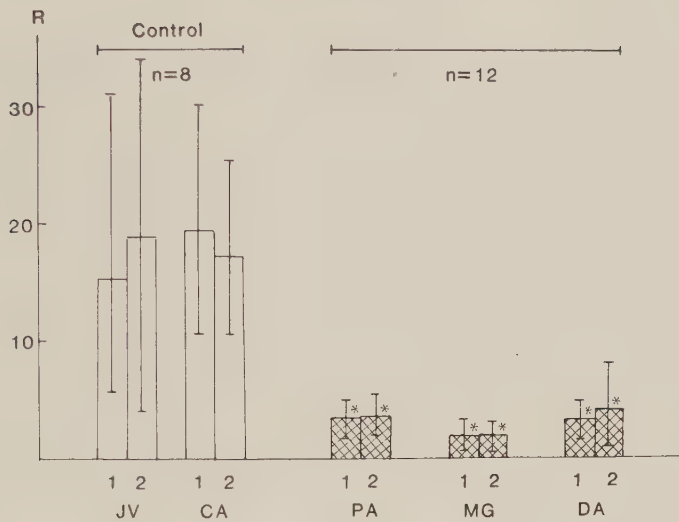


FIG. 4.

6-keto-PGF_{1α}/TxB₂ ratio (R) in grafts removed after 90 days. Mean ± 95 % confidence of the mean. For code, see Figs. 1 and 2.

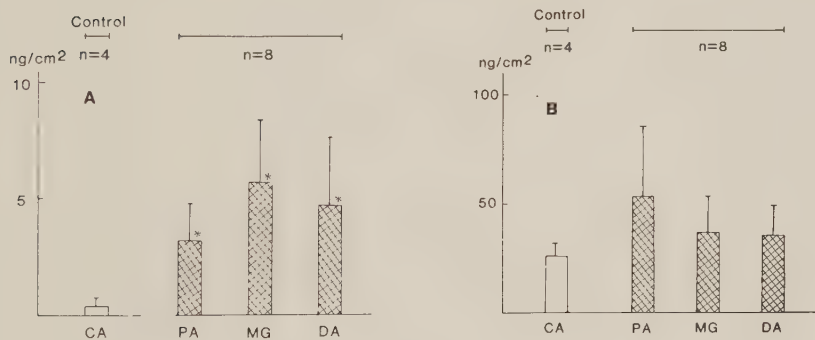


FIG. 5

TxB₂ (A) and 6-keto-PGF_{1α} (B) synthesis in grafts removed after 10 days. First 15 min incubation period. For code, see Figs. 1 and 2.

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THE EFFECT OF INHIBITION OF THROMBOXANE SYNTHESIS IN
EXPERIMENTAL THROMBOSIS AND HEMOSTASIS.

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ABSTRACT

The effect of a selective inhibitor of thromboxane synthesis (dazoxiben) was evaluated in different acute models for thrombosis and hemostasis. Dazoxiben significantly reduced the thrombogenicity of the modified human umbilical vein (Dardik Biograft) inserted in the carotid artery position in sheep. The effect was evident concerning patency, thrombus weight and platelet accumulation at the distal anastomosis. This paralleled a decreased production of thromboxane in both anastomoses and the midgraft region. Dazoxiben did not reduce either the frequency of jugular vein thrombosis (induced by a combination of endothelial damage and flow restriction) or arteriolar microembolism after laser injury in rabbits. Neither did it influence initial hemostasis as evaluated by measuring the hemostatic plug formation in the rabbit mesenteric microcirculation. It is concluded that thromboxane synthesis inhibition may be of value when attempting to improve the performance of small diameter vascular prostheses, the data obtained indicating a low risk for hemorrhagic complications.

Key words: Thromboxane synthesis inhibitor, thrombosis, hemostasis, graft thrombogenicity.

INTRODUCTION

The balance between prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) has aroused much interest (1), especially in connection with the pathogenesis of arterial thrombosis. Prostacyclin, synthesized from endoperoxides in the vascular endothelium, is a potent inhibitor of platelet aggregation. Thromboxane A_2 which is synthesized in different cells, especially in platelets, induces platelet aggregation. The synthesis of both is inhibited by cyclooxygenase inhibitors such as nonsteroidal antiinflammatory substances. From theoretical considerations, it can be postulated that a selective inhibitor of thromboxane synthesis should be more beneficial in counteracting thrombus formation than a cyclooxygenase inhibitor. In a previous study, we found that the ratio of 6-keto- $\text{PGF}_{1\alpha}$ to TxB_2 , the stable end products of prostacyclin and TxA_2 respectively, was strongly decreased in the pseudointima of umbilical vein grafts inserted into carotid arteries of sheep (2). It therefore seemed logical also to evaluate the possible acute effects on thrombosis and haemostasis of a selective thromboxane synthesis inhibitor and the compound chosen was dazoxiben, an imidazol derivative.

MATERIAL AND METHODS

Arterial graft thrombus formation

The experimental model has been previously described in detail (3). Ten adult sheep of both sexes (weights 30-40 kg) were used and anesthetized with sodium thiobarbital (Pentotal^R) using controlled ventilation with 20 % oxygen. The right femoral artery was cannulated for blood sampling and pressure recordings. Both common carotid arteries were isolated, all tributaries being ligated. The vessels were then placed in a specially-designed holder made of aluminium, heated to body temperature by circulating warm water. The background radiation levels on both sides were determined and then autologous platelets labelled with 30 MBq of ^{32}P were infused. The levels of radioactivity in the vessels quickly reached "steady state", but were usually followed for 30-45 minutes to determine whether or not "trapping" had occurred. The holder was then removed, a segment of the carotid artery resected and a 6 mm inner diameter glutaraldehyde-tanned human umbilical vein graft (Dardik Biograft, Meadox) inserted, using two end-to-end anastomoses and running sutures with 7:0 polypropylene. The graft length was 6 cm. Flow was restored on both sides simultaneously. After hemostasis, the vessels were replaced in the holders and the measurements of radioactivity were continued at four points: proximal and distal anastomosis, midgraft and a reference point in the artery proximal to the graft. To accelerate the thrombotic process, flow was restricted to 25 ml/min. by placing a special clamp distal to the graft. Flows were measured electromagnetically (Carolina Medical Electronics Inc., USA). The counting-rates at each of the four points were measured in cycles, about once every 15 minutes over a four-hour period. The grafts were then removed, cut open lengthwise and inspected for thrombotic material, which was removed and immediately weighed. In cases of thrombotic occlusion, time to occlusion was noted. Six sheep were used as controls and

four were given dazoxiben hydrochloride, 4-[2-(1H-imidazol-1-yl)ethoxy] benzoic acid hydrochloride. In all, 25 mg/kg body-weight was administered as an intravenous infusion of 10 mg/kg body-weight while inserting the grafts, i.e., about 60 min. before restoring blood-flow, and 15 mg/kg body-weight immediately prior to restoration of flow. In two of the control and one dazoxiben treated sheep, the experiments were performed without using labelled platelets, only thrombus weight and patency being recorded. However, the grafts were placed in the holders during the perfusion period, in order not to alter the flow conditions.

Ex vivo studies

After opening the grafts and removing thrombotic material and periprosthetic tissue, sections (0.5-1.0 cm²) from the anastomotic sites including equal portions of the artery and the graft and mid-graft regions were tested for production of 6-keto-PGF_{1α} and TxB₂.

Incubation of the sections. The specimens were immediately rinsed in ice-cold Ringer's solution at a pH of 7.4. Any gross thrombus material was carefully removed and the specimens superfused with Ringer's solution. They were then placed in 1 ml of fresh Ringer's solution and incubated for 15 min. at 37°C. Aliquots of the supernatant were frozen at -70°C until TxB₂ and 6-keto-PGF_{1α} assays were performed.

The production of 6-keto-PGF_{1α} and TxB₂ was determined by radioimmunoassays (RIAs), using labelled antigen and antirabbit antiserum as antibodies (6-keto-prostaglandin F_{1α} ¹²⁵I RIA Kit and thromboxane B₂ ¹²⁵I RIA Kit, New England Nuclear). Each sample was assayed in duplicate.

Histological methods. Midgraft sections adjacent to those taken for radioimmunoassay were fixed in 10 % neutral formaldehyde. Sections 5 μm thick were stained with hematoxylin-eosin and with the picro-Mallory method for detecting platelets.

Venous thrombus formation

Fifteen rabbits of both sexes (weight 2.5-4.9 kg) were used and thrombus formation was induced by a combination of endothelial injury and flow reduction, basically using the method described by Peterson & Zucker (4) as modified by Björck et al (5). Both jugular veins were exposed through a transverse cervical incision. A one cm long segment of each vein was isolated between clamps, flushed with Ringer's lactate through a 27 gauge cannula and then filled with ethoxysclerol (Kreussler & Co, Wiesbaden-Biebrich, Germany). After 5 minutes the segment was again flushed with Ringer's lactate, clamps removed and flow restored but with a ligature proximal to the segment, restricting the lumen to the width of an 0.8 mm cannula. After 30 minutes, the vein was transected just distal to the constricting ligature and the segment inspected for presence of thrombosis. If, although there was a thrombus present, the proximal end bled, the thrombus was considered non-occlusive. The thrombus was immediately weighed.

Five rabbits were given saline (4 ml) and ten dazoxiben intravenously. Five animals received 10 mg/kg, 5 mg/kg given 15 minutes before clamping and 5 mg/kg at flow restriction. Five animals were given 25 mg/kg, 10 mg/kg 15 minutes before clamping and 15 mg/kg at flow restriction. Dazoxiben was dissolved in saline and injected in a volume of 4 ml.

Rabbit vein sections (0.2-1.0 cm²) from non-injured veins and veins injured with ethoxysclerol were tested for production of 6-keto-PGF_{1α} and TxB₂ according to the methods described above.

Platelet activity following laser injury

Six Sandy-Lop rabbits of both sexes with an average weight of 4.0 kg were used. Titanium ear chambers were inserted 60 days prior to the investigation resulting in suitable ear chambers with adequate regenerative tissue composed of arterioles, venules and lymphatics. After a laser injury to arterioles measuring 20-40 μ in diameter, a clump of red blood cells adhered to the endothelium at the site of injury. Platelets then began to aggregate forming a microthrombus which embolized either in fragments or as whole (6). This phenomenon continued for several minutes and the cumulative number of emboli over a 10 minute period was recorded. Two determinations were obtained before treatment, and at 15, 90 and 180 minutes after dazoxiben administration. Dazoxiben in a dose of 4 mg/kg in 1 ml saline was injected intravenously after the control determinations.

Microvascular hemostasis

Ten New Zealand rabbits of both sexes with weights ranging from 2.0 to 2.2 kg were studied. They were anesthetized with sodium pentobarbital (Mebumal^R). The microvasculature in the transparent areas of the mesentery was observed through an intravital microscope (Leitz Biomed - magnification 81x). The exteriorized mesenteric segment was continuously superfused with Tyrode's solution at 38° C. Five arterioles and five venules measuring 20-40 μ in luminal diameter were transected before and after administering the drug. The bleeding times from the proximal arteriolar segment and from either the proximal or distal venular segments were recorded as follows: primary hemostatic plug formation time (PHT), corresponding to the time between transection and the first arrest of bleeding, and the total hemostatic plug formation time (THT) which included the PHT and the sum of all rebleeding times until there was no recurrence over a 10 minute period. The frequency of rebleeding was also recorded. A detailed description of the method has been published elsewhere (7). Dazoxiben was given as a continuous intravenous infusion in a dose of 25 mg/kg body-weight in 10 ml saline during 60 minutes, starting 10 minutes before experiments.

Statistical methods

The differences in thrombosis frequency were analysed using Fisher's exact test and the significance of the measured values of thrombus weight and platelet accumulation determined using the Mann-Whitney U-test. In analysing the differences in 6-keto-PGF_{1α} and TxB₂, Student's t-test and Wilcoxon's ranksum test for

paired observations were used. The hemostatic plug formation times showed a skew distribution and hence were converted to a more normal distribution by a logarithmic transformation. The significance of differences was evaluated using Student's paired t-test. Differences were considered to be significant when $p < 0.05$.

RESULTS

Only one of twelve Biografts in control sheep remained patent throughout the four-hour period studied. After dazoxiben treatment six of eight grafts remained open, which is significantly more than in control sheep ($p < 0.01$). The mean time until occlusion was 45 minutes (15-135) in untreated animals and 113 minutes (105 and 120) in dazoxiben treated ones. The mean thrombus weight was 646 ± 292 mg in untreated and 265 ± 191 mg in dazoxiben treated sheep ($p < 0.01$).

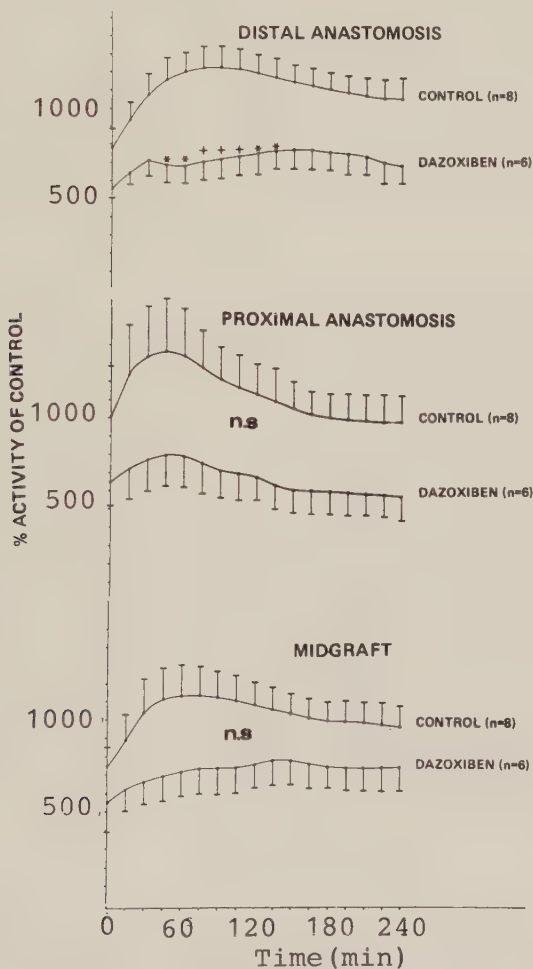


FIG. 1. Comparison of ^{32}P -platelet accumulation in the different regions of Biografts in control sheep and animals receiving dazoxiben (25 mg/kg i.v.) $M \pm \text{SEM}$. * $p < 0.05$ + $p < 0.02$.

Fig. 1 shows the percent changes in platelet activity relative to the reference point in the artery. The activities in dazoxiben-treated animals were always lower than in untreated sheep but significant differences were only obtained in the distal anastomosis between 45 and 135 min. after restoring blood flow. Several animals in the dazoxiben-treated group showed rather rapid changes in counting-rates at the distal or proximal anastomoses. Results for a typical case are shown in Fig. 2.

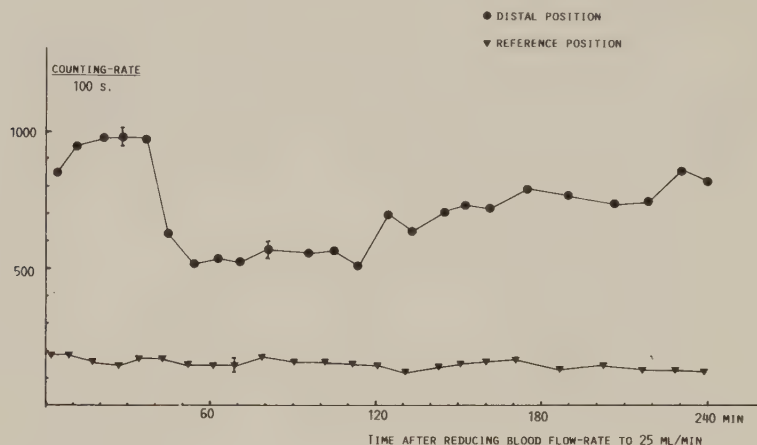


FIG. 2. Counting rates in 100 s counting-intervals for the distal anastomosis and reference point in a dazoxiben-treated animal. The statistical errors for selected points are shown.

Dazoxiben treatment resulted in a significantly decreased production of TxB_2 in both anastomotic, and in the mid-graft regions (Fig. 3). Grafts from sheep treated with dazoxiben also showed a tendency to increased levels of 6-keto-PGF $_{1\alpha}$ in the anastomotic regions (Fig. 4), although this was not statistically significant. Histologically, scattered monocytes were found on the surfaces of the grafts while platelet aggregates were absent.

Table I shows that the frequency of jugular vein thrombosis was the same in rabbits treated with dazoxiben and with saline. The median weight of thrombi in dazoxiben (10 mg/kg) treated rabbits was 13 (6-20) mg as against 46 (7-131) mg in saline treated, this difference being significant ($p < 0.05$). Rabbits receiving 25 mg/kg dazoxiben showed no reduction in mean thrombus weight 47 (7-90) mg.

TABLE I. The effect of dazoxiben on the formation of jugular vein thrombi.

	Number of thrombi		
	0	1	2
Saline (number of animals)	1	3	1
Dazoxiben 10 mg/kg (number of animals)	1	1	3
Dazoxiben 25 mg/kg (number of animals)	0	0	5

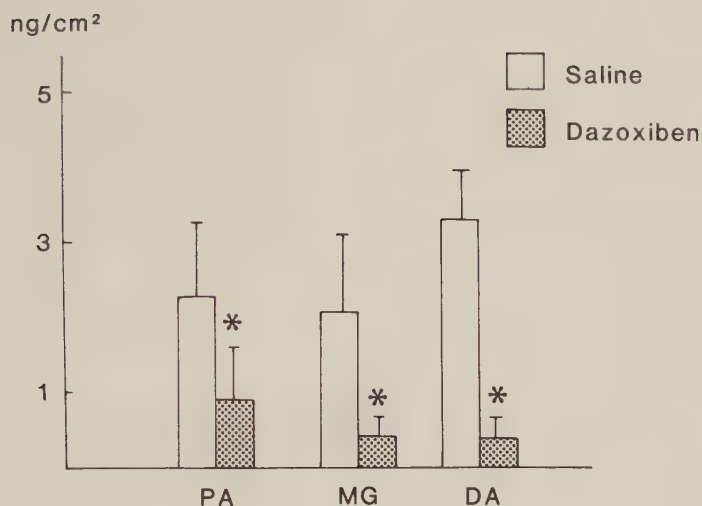


FIG. 3

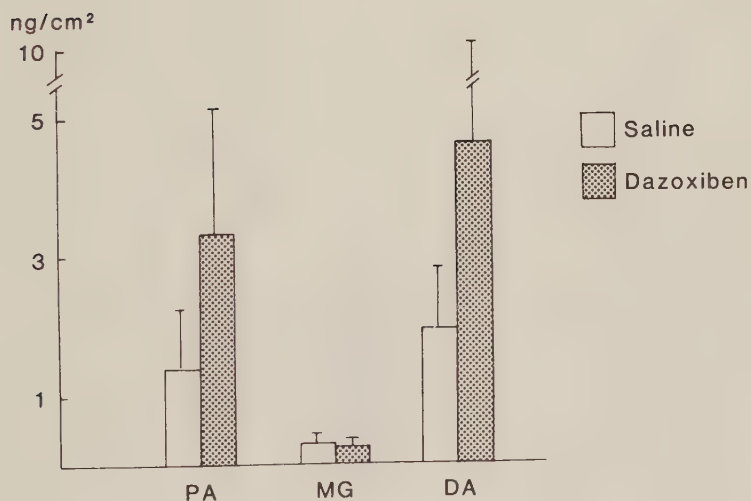


FIG. 4.

FIGS. 3-4. Production of TxB_2 (Fig. 3) and $6\text{-keto-PGF}_{1\alpha}$ (Fig. 4) in Biografts placed in the carotid artery of sheep. PA=proximal anastomosis, MG=mid-graft, DA=distal anastomosis, $n=4$ (saline) and 8 (dazoxiben, 25 mg/kg), *=significantly different from saline.

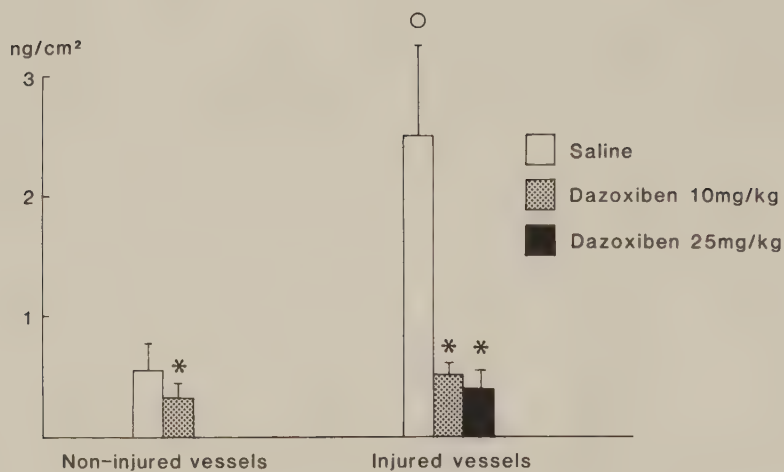


FIG. 5

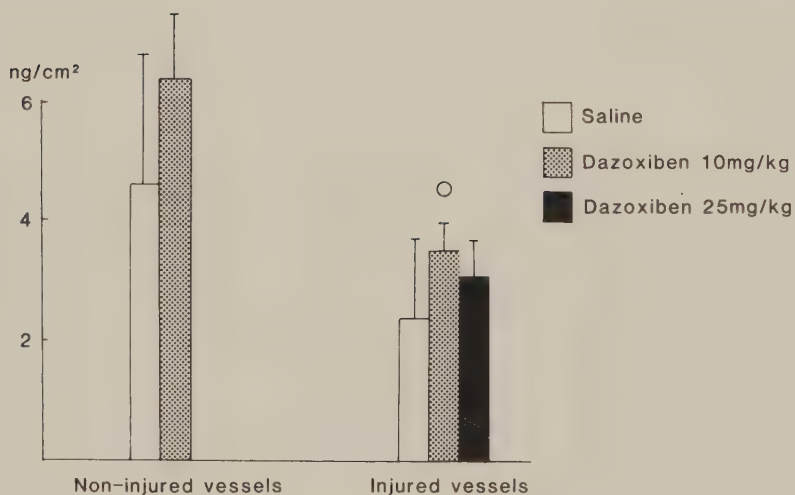


FIG. 6

FIGS. 5-6. Production of TxB_2 (Fig. 5) and 6-keto- $\text{PGF}_{1\alpha}$ (Fig. 6) in normal jugular veins and jugular veins injured by ethoxysclerol. $n=8$. *=significantly different from saline. 0=significantly different from non-injured vessels.

Rabbit veins injured with ethoxysclerol showed an increased production of TxB_2 compared to normal veins from the same rabbit (Fig. 5). Treatment with dazoxiben decreased the production of TxB_2 to the level found in non-injured vessels. There was a tendency for decreased production of 6-keto-PGF $_{1\alpha}$ in injured vessels, this decrease being significant in dazoxiben-treated rabbits (Fig. 6). In rabbits treated with dazoxiben there was a tendency towards an increased production of 6-keto-PGI $_{1\alpha}$ compared to nontreated animals but this increase was not statistically significant.

From Figure 7 it is seen that the frequency of laser induced microvascular embolism was not influenced by dazoxiben administration.

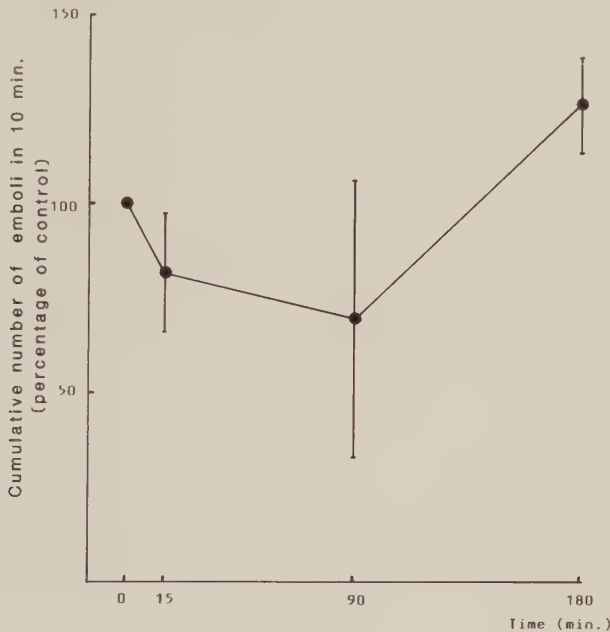


FIG. 7. The effect of dazoxiben on the cumulative number of emboli after arteriolar laser injury in six rabbits. Dazoxiben (4 mg/kg) was given immediately after the 0 determinations.

Table II shows a slight prolongation of hemostatic plug formation times in both arterioles and venules after dazoxiben administration but the difference was not statistically significant.

TABLE II. The influence of dazoxiben (25 mg/kg in continuous infusion) on total (THT) and primary (PHT) hemostatic plug formation time (sec.). Mean $\pm$ SD.

	Arterioles		Venules	
	THT	PHT	THT	PHT
Before treatment	61 $\pm$ 34	56 $\pm$ 29	240 $\pm$ 178	205 $\pm$ 134
After dazoxiben	81 $\pm$ 53	74 $\pm$ 50	349 $\pm$ 286	217 $\pm$ 137

Dazoxiben is an imidazol derivative which selectively inhibits thromboxane synthesis (8-10). Both arachidonic acid and collagen induced platelet aggregation is inhibited (9,11) whereas ADP induced is not (9). An in vivo protective effect at least on arterial thrombosis has been reported (12-14), but platelet accumulation in arteriovenous dacron grafts in baboons (15) or in dacron grafts in an artificial circulation simulating femoropopliteal bypass (16) could not be reduced by dazoxiben treatment. The doses used in our study, seem to be adequate to judge from the experimental data in the studies mentioned (also 4 mg/kg) and from our in vitro results. It has moreover been suggested that thromboxane synthesis inhibitors may act in vivo as antiaggregating compounds by potentiating the activity of prostacyclin (17) or enhancing the responsiveness of platelets to endogenous prostacyclin (18).

An unambiguous and significant effect of dazoxiben in this study was the reduction of the acute thrombogenicity of Biografts inserted in the carotid position in sheep. This coincided with a significant decrease of TxB_2 production. The results of the graft experiments lead to the conclusion that thromboxane release may be important in the pathogenesis of acute graft thrombosis. Blood cells, trapped on the vessel surface, may have been responsible for the production of TxB_2 . Platelets are believed to be the main source of TxB_2 in most species (19), but other cells as fibroblasts, leucocytes, smooth muscle cells and endothelial cells have also been shown to produce TxB_2 (20-23). However, sheep platelets have been reported to produce only minute amounts of TxB_2 (24), and in addition scattered monocytes but no platelets were found on the graft surfaces. It is thus possible that monocytes were the main source of TxB_2 , particularly since these cells are known to be rich in arachidonic acid (25). In a previous study, a higher thromboxane production in Biografts than in adjacent arteries has been demonstrated (2). In addition, it was possible to increase the 6-keto-PGF $_{1\alpha}$ / TxB_2 ratio in vascular tissue with dazoxiben (26). This pharmacologic treatment resulted in a patency rate of the same magnitude as when the graft surface was treated with heparin and alcohol before insertion (27,28).

Interesting findings in this study were the rather rapid decreases of platelet activity after the initial rise at the distal and proximal anastomoses in some grafts in dazoxiben treated sheep. This is almost certainly due to embolization and may indicate that thrombi produced in the presence of dazoxiben are more fragile than normal. Although the frequency of venous thrombosis was not influenced by dazoxiben, the diminished thrombus weight in some veins could also be an expression of an altered thrombus structure.

The increased production of thromboxane in injured rabbit veins could be due to trapped inflammatory cells and/or increased production in the cells of the vessel wall since it has been shown earlier that injury with balloon catheter increases the production of TxB_2 (29). In the latter study, however, prostacyclin production was even more enhanced unlike the findings in

the present investigation where there was a tendency towards decreased production of 6-keto-PGF_{1α} in injured vessels. This difference might possibly be accounted for by the more pronounced injury or even destruction of the prostacyclin producing endothelium caused by ethoxysclerol in the present investigation.

Although there was a reduction in venous thrombus weight at the lower dazoxiben dose, the frequency of thrombosis was not decreased. This observation seems to favour the opinion that platelet aggregation is of minor importance in the initial development of venous thrombi in this model (30), the balance between prostacyclin and thromboxane not being as important as it is on the arterial side. Using a similar model for thrombus induction, it was not possible to show effects with other platelet inhibitors (31), whereas substances influencing coagulation and fibrinolysis do reduce the frequency of thrombosis (5,32).

There is a large amount of evidence that hemostatic plug formation and laser induced aggregation are platelet responses primarily induced by ADP and not by collagen (33-36). It is therefore not surprising that dazoxiben does not appear to influence these reactions since it does not inhibit ADP induced aggregation in vitro (9,10).

In conclusion, this study showed that umbilical vein grafts inserted into carotid arteries in sheep produced thromboxane. This production was reduced by a selective thromboxane synthesis inhibitor. Treatment with this inhibitor significantly reduced the acute graft thrombogenicity. Although an inhibition of thromboxane production was noted also in injured vein walls the frequency of venous thrombosis was not influenced, indicating that thromboxane is of minor pathogenetic importance in the development of venous thrombosis. Initial hemostasis was uninfluenced by inhibition of thromboxane synthesis. The concept of thromboxane synthesis inhibition may thus be of value when considering attempts to improve the performance of small diameter arterial prostheses, the experimental data indicating a low risk for hemorrhagic complications.

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